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Oxidative stress triggers pancreatic acinar cell apoptosis and an inflammatory response

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ABSTRACT

English

Recent studies have recognized the role of reactive oxygen species (ROS) in pancreatic inflammation and carcinogenesis. However, no in vivo animal model has been described so far. Caerulein and ethanol have been reported to induce oxidative stress in pancreatic acinar cells. Thus, we tested the efficiency of ROS-generation by combining caerulein and ethanol treatment in pancreatic inflammation and analyze themechanism in detail.

Deutsch

Neue Studien belegen, dass reaktive Sauerstoffverbindungen (ROS) bei der Entzündung und Pankreaskarzinogenese eine wichtige Rolle spielen. Dazu wurde allerdings noch kein geeignetes Tiermodel entwickelt. Es konnte jedoch gezeigt werden, dass Caerulein und Ethanol oxidativen Stress in pankreatischenAzinuszellen auslösen kann. Daher haben wir im Tiermodel die Effektivität einer Caerulein/Ethanol Behandlung zur Induktion von ROS-generiertem Stress getestet und den Mechanismus untersucht.

1. INTRODUCTION

1.1. Reactive oxygen species (ROS) and carcinogenesis

1.1.1. Source of ROS and redox environment of a cell

Chemically reactive molecules containing oxygen is so called reactive oxygen species (ROS) which can be classified as a form of free radicals like oxygen ions or non-radicals format as peroxides. ROS form as a natural byproduct of the normal metabolism of oxygen and is known to be mutagenic and plays a role in cancer formation (Shibutani et al., 1991). ROS formation, which can be derived from exogenous and endogenous sources, is a natural consequence of aerobic metabolism (Castro and Freeman, 2001) (Figure 1).As for the exogenous sources, the environmental agents such as radiation, metal and chemicals that are directly metabolized to radicals or promote ROS generation in living cells (Bonney et al., 1991; Dreher and Junod, 1996; Halliwell and Aruoma, 1991; Jaruga and Dizdaroglu, 1996; Marnett, 2000; Wang et al., 1998). Mitochondrion is the major site for energy production and cellular respiration which produce approximately 98% of endogenous source of ROS (Freeman and Crapo, 1982). Under physiological circumstances, the mitochondrion is an intracellular organelle which is responsible for energy production by cellular respiration. However, the leaking electron from mitochondrial electron transport chain eventually interacts with oxygen to produce superoxide radicals (Benzi et al., 1992; Brookes et al., 2002; Castro and Freeman, 2001; Hanukoglu et al., 1993; Kinnula et al., 1995; McCord, 2000; Salvador et al., 2001). In order to detoxify foreign compounds, the oxygen is required by cytochrome P450 in the endoplasmatic reticulum for oxidation. Besides, P450 is also involved in hydroxylation reactions. Thus, superoxide radicals form during both oxidation and hydroxylation reactions (Butler and Hoey, 1993). In addition, enzymes within the peroxisomes use molecular

oxygen to remove hydrogen atoms, forming H_2O_2 during the peroxisomes oxidation of fatty acids (Klaunig and Kamendulis, 2004). Furthermore xanthine oxidase (XO), as a highly versatile enzyme within cytoplasm of a cell produce superoxide anion and hydrogen peroxide while it catalyzes the hydroxylation of purines and catalyzes the reaction of hypoxanthine to xanthine and xanthine to uric acid (Li and Jackson, 2002; Valko et al., 2004). It is also worth mentioning that under abnormal circumstances as inflammation, NADPH oxidase in the membranes of cells such as neutrophils, eosinophils and macrophages generates superoxide by transferring electrons from NADPH inside the cell across the membrane and coupling these to molecular oxygen to produce superoxide anion which will undergo further reactions to generate ROS (Conner and Grisham, 1996).

Under normal physiological conditions, intracellular ROS are constantly generated and eliminated and are required to drive stable regulatory pathways via the scavenging system (Figure 2). Non-enzymatic antioxidants act as one of the two important parts of the intracellular “redox buffering” capacity and is substantiated primarily by Vitamin C, Vitamin E, carotenoids and thiol antioxidants (glutathione, thioredoxin and lipoic acid) (McCall and Frei, 1999).These small molecule antioxidants act similarly to peroxyl radical scavengers because they directly or indirectly combine and degrade free radicals into other harmful formations to maintain a stable cell redox state as a hydrogen donor (such as vitamin C). The most efficient enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxides (GPX) (Mates et al., 1999). SOD catalyzes most intracellular ROS to O_2 or the less–reactive species, H_2O_2, which is finally decomposed to water by catalase and GPX. Eventually, the majority of ROS-detoxifying enzymes require reduced glutathione (GSH) activity, which relies on NADPH. Therefore, NADPH provides the ultimate reducing power for these biological reactions. Taken together, both non-enzymatic antioxidants and enzymatic antioxidants act as an “antioxidant network” that maintains a suitable cell redox balance (Sies et al., 2005).

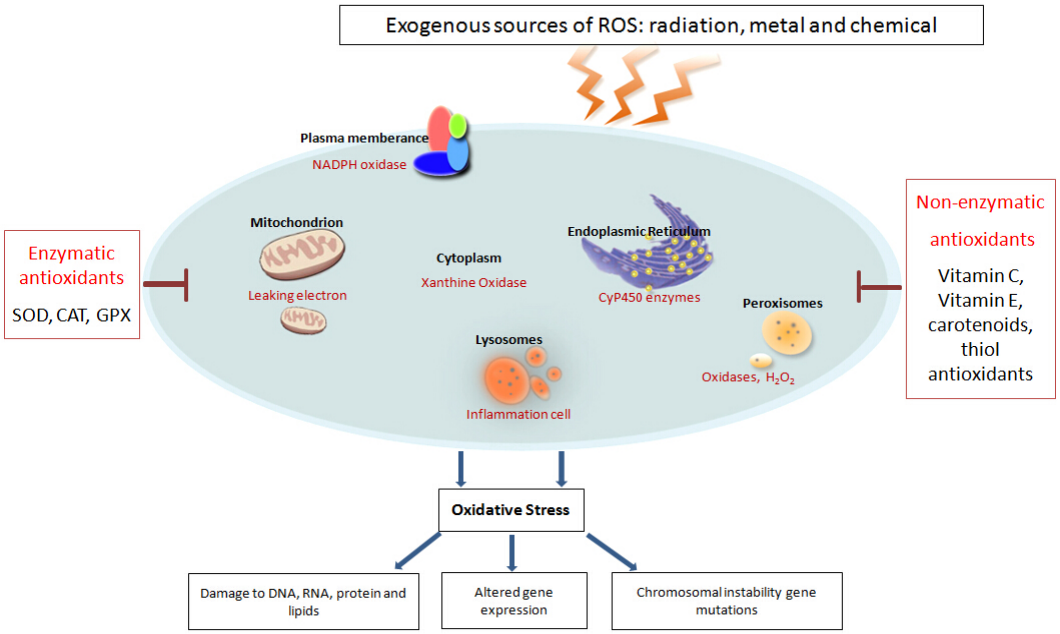

Figure 1: Source of the ROS, ROS scavenger and their role in cancer carcinogenesis

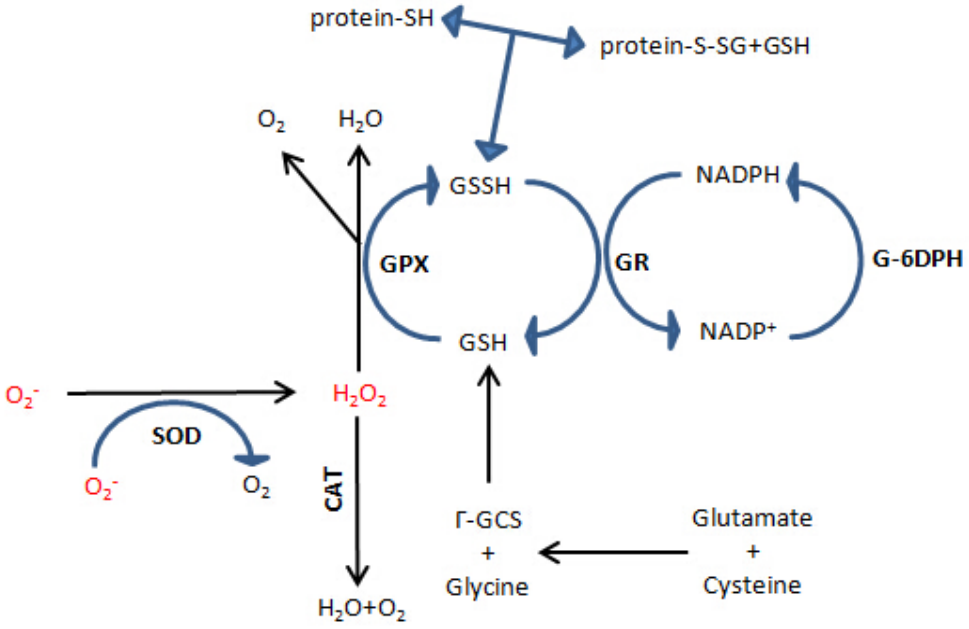

Figure 2: The ROS scavenger system

1.1.2. The role of oxidative stress in cancer carcinogenesis

In general, carcinogenesis is noted as a complex multiple and distinct process with different underlying mechanisms that gradually leads a healthy cell to a precancerous state and finally to an early stage of cancer (Klaunig and

Kamendulis, 2004; Trueba et al., 2004). Thus, the multi-stage cancer development process is characterized by the cumulative action of multiple events occurring in a single cell and includes the following three stages: initiation, promotion and progression, each stage is influenced by ROS generation (Klaunig and Kamendulis, 2004). Briefly, oxidative DNA damage occurs via ROS activity. As a result, a non-lethal DNA mutation (strand breaks, base modifications and DNA–protein cross linkages etc. (Kim et al., 2006) occurs in theinitial stage and produces altered dividing cells that temporarily interrupt their cell cycle at stage G1, S, or G2 to repair the DNA damage and resume division (Kohen and Nyska, 2002; Valko et al., 2006). During the promotion stage, the continuous presence of the tumor promotion stimulus causes cell proliferation induction and/or inhibition of programmed cell death to selective clonal expansion of the initiated cells. Many tumor promoters strongly affect cellular antioxidant defense systems, a cytotoxic level of oxidative stress induces cell apoptosis or even necrosis, and a low level of oxidative stress stimulates cell division to enter the promotion stage (Dreher and Junod, 1996). The final stage of carcinogenic process is characterized by the accumulation of additional genetic damage, which leads to genetic instability and the disruption of chromosome integrity (Klaunig and Kamendulis, 2004).To this end, DNA alterations caused by ROS are removed by specific and non-specific repair mechanisms, while the misrepair of DNA damage results in mutations, leading to carcinogenesis (Dreher and Junod, 1996; Marnett, 2000). Overall, when ROS production and detoxification are not properly controlled in the cellular environment, the regulation of downstream molecular events (numerous oncogenes and tumor suppressor genes) impinge on proliferation, apoptosis and cell migration, all of which are connected with cancer progression (Figure 3).

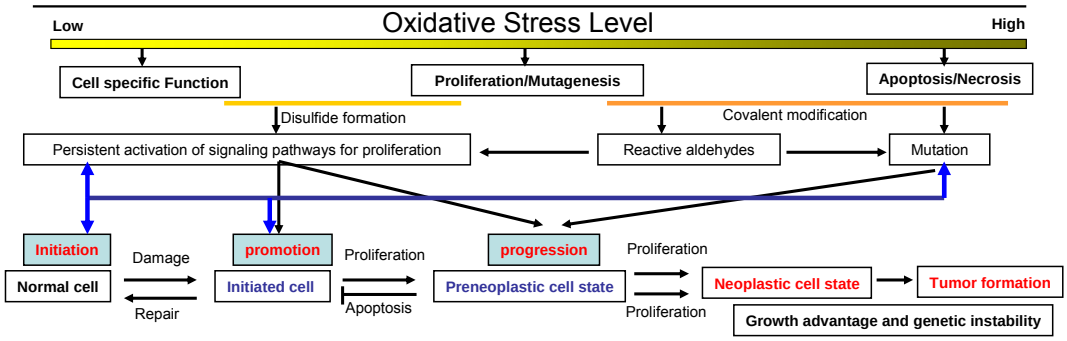

Figure 3: The significance of different level of oxidative stress in the three stages model of carcinogenesis

1.2. Pancreatitis, ROS and pancreatic cancer

Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive tumor that has the highest case-fatality rate of any major cancer with an overall 5-year survival rate of less than 5%. PDAC is the fourth leading cause of cancer death in the USA with over 45,200 estimated new cases in 2013 (Siegel et al., 2013). Unfortunately, the etiology of PDAC is not yet fully understood.

1.2.1. Inflammation, ROS and pancreatic carcinogenesis

Numerous studies have aimed to identify genetic changes and the role of these alterations in pancreatic cancer development. However, comparatively few research studies have focused on the role of inflammation and reactive oxygen species (ROS). Inflammation, together with ROS generation may represent an early stage of pancreatic malignant transformation as a result of an abnormal microenvironment with subsequent genetic changes occurring as a later manifestation of the prolonged inflammatory response. In addition, the so-called “LANDSCAPER THEORY” proposes that pancreatitis/inflammation mediated by cytokines, ROS and upregulated pro-inflammatory pathways produce a defective population of cells from normal stromal tissue. Abnormal microenvironments include factors that promote genomic damage (tumor suppressor gene mutations

or oncogene activation) and unregulated growth. The combination of cell damage and proliferation may promote the production of malignant transformed cells, and the uncontrolled proliferating of transformed cells may finally lead to the formation of cancer. Recently, more studies have shown that inflammation together with ROS production and the induction of the cell cycle may play a contributory role in numerous pathologies, including early stage pancreatic carcinogenesis (Goto et al., 1991; Greer and Whitcomb, 2009; Whitcomb, 2004). However, the exact inflammation mechanism that leads to pancreatic malignancy is not well understood.

Nevertheless, among all these theories, the probable inflammatory mechanisms involved in pancreatic cancer development are described as follows.

First, during pancreatitis, the release of cytokines together with ROS produces inflammation and cellular damage (Norman, 1998). Acute pancreatitis healing restores the pancreatic tissue to normal function; however, inflammation in chronic pancreatitis persists and healing occurs simultaneously, which produces high levels of ROS. The injured acinar is believed to recur, causing prolonged inflammation with a persistent infiltration of immune cells, leading to fibrosis (Steer et al., 1995). Although convincing evidence demonstrated by a number of epidemiologic studies has shown that pancreatic cancer arises from all forms of chronic pancreatitis, whether or how ROS is produced and mediates the pancreatic malignancy procedure remains unclear.

Second, during pancreatitis, various pathways and transcription factors are activated, including the NF-κB pathway. In normal cells, NF-κb dimers bind to inhibitory IκB proteins (IκBa, IκBb and IκBe) and remain in an inactive state in the cytoplasm. During pancreatitis, the activation of NF-κB leads to cancer progression by regulating the expression of genes involved in cell growth and proliferation, anti-apoptosis, angiogenesis and metastasis (Antunes and Han,

2009; Hagemann et al., 2009; Kim et al., 2009; Liu et al., 2006; Mauro et al., 2009). ROS also directly influences NF-κB activity (Hirota et al., 1999).

Finally, ROS, acting as the initiator as well as the byproducts during inflammation, cause DNA strand breaks, sister chromatic exchanges, mutation, and DNA adduct formation. Furthermore, the most frequent mutation caused by ROS is a p53 mutation, suggesting that ROS cause the loss of tumor suppressor function (Jackson and Loeb, 2001; Palli et al., 1997). Under oxidative conditions, p53 is functionally destabilized or mutated, which leads to the proliferation of the cells with DNA damage (Hussain et al., 2000).

In summary, pancreatic inflammation, mediated by cytokines, ROS, and up-regulated pro-inflammatory signal pathways may play a key role in the early development of pancreatic malignancy.

1.2.2. mTOR, TSC1-TSC2 complex, p53 and ROS

The mammalian target of rapamycin (mTOR) integrates a variety of intracellular and extracellular cues, including growth factors, energy stress, amino acids and oxygen. mTOR has emerged as a key regulator of cellular metabolism. mTOR controls mitochondrial oxidative function through a YY1–PGC-1α transcriptional complex (Cunningham et al., 2007). Up-regulated mTOR activity is associated with a higher level of intracellular reactive oxygen species (ROS) (Jang and Sharkis, 2007). TSC was first defined by genetic mutations leading to nonmalignant growth in solid organs in patients with genetic lesions of two genes, now called *TSC1* and *TSC2* (European Chromosome 16 Tuberous Sclerosis, 1993; Khalifa et al., 2014). More recently, TSC has emerged as the major negative regulator of mTOR (Melnik and Schmitz, 2013). The generation of ROS activity in vivo affects TSC-mTOR pathway, leading to increased mitochondria biogenesis, which is also largely restored by repressing ROS production (Chen et

al., 2008). Taken together, the TSC-mTOR pathway is important mediators of ROS; therefore, the TSC-mTOR pathway may performs a largely underestimated role in pancreatic carcinogenesis.

p53 is a sequence-specific transcription factor as well as a critical tumor suppressor gene most frequently mutated in human cancer (Levine, 1997). p53 regulates genes implicated in a variety of cellular processes such as cell cycle arrest, DNA repair, apoptosis, senescence, autophagy, metabolism, oxidative/redox stress, angiogenesis and many more relevant processes associated with normal physiology and pathology (Brady and Attardi, 2010). In addition to acting as a transcription factor, p53 also acts directly to regulate cell fate (Chipuk et al., 2005; Chipuk et al., 2004; Mihara et al., 2003). Nevertheless, how ROS modulate selective transactivation of p53 target genes a challenging question that remains to be clarified. As a tumor suppresser, one of the major functions of p53 is to restrict abnormal or stress-exposed cells before DNA damage is caused by ROS (Lane, 1992). Multiple cellular processes exist in which both ROS and p53 are involved. In response to ROS that leads to DNA damage, wild-type p53 directs cells either to cell cycle arrest, senescence, or apoptosis via the differential activation of target genes (Dionisi et al., 1975). In addition, under normal circumstances, p53 inactivates mTORC1 together with TSC function through a negative feedback signal loop that helps cells cope with oxidative stress. Thus, the dysfunction to one or two points in the loop may imbalance the feedback mechanism, leading to an unpredictable cell fate. A recent study showed that the redox-mediated regulation of p53 may be involved in controlling the cellular switch between survival and death. The interplay between p53 and mitochondrial antioxidant enzyme manganese superoxide dismutase (MnSOD) may be the mechanism by which p53 coordinates their response to stress and regulate ROS levels (Sun et al., 2013). Anna A. Sablina et al. demonstrates that p53 down-regulation elevates intracellular ROS and that p53 deficiency promotes DNA oxidation and mutagenesis. Therefore, increased

intracellular ROS in p53-deficient mice contributes to the cancer-prone phenotype (Sablina et al., 2005). Other studies have shown that p53 suppression results in a significant decrease in the basal antioxidant genes without affecting the expression of the pro-oxidant genes BAX, NQO1, and PUMA (Sablina et al., 2005), thus leading to an increase in ROS and subsequently to DNA oxidative damage,which causes carcinogenesis. A recently published paper by Al Saati T et al. showed that the expression of tumor protein p53-induced nuclear protein 1 (TP53INP1), which is involved in p53 phosphorylation, is lost at the pancreatic intraepithelial neoplasia 1b (PanIN1b)/PanIN2 stage of pancreatic carcinogenesis. TP53INP1 deficiency increases cellular ROS levels and accelerates PanIN formation, highlighting the importance of TP53INP1 in the control of oxidative status during pancreatic cancerdevelopment (Al Saati et al., 2013).

1.3. Experimental animal model for inducing oxidative stress

The pancreas is important in the digestion process, and pancreatitistogether with ROS creation play a contributory role in numerous pathologies; therefore, various in vivo animal models have been introduced to modulate either acute or chronic pancreatitis since the 18th century. For a systematic presentation of the current animals, grouping them according to the type of inflammation stimulus used is useful. Several models combine two or three treatments; therefore, the models are classified by the type of trigger employed. The most popular models include ethanol feeding, repetitive cerulean injections and surgical ligation of the pancreatic duct. Recently, several genetic models or immunological models that also induce pancreatic inflammation have been reported.

1.3.1. Alcohol pancreatitis and caerulein pancreatitis

Alcohol abuse commonly occurs; however, epidemiological evidence indicates that excessive alcohol uptake is a primary cause of acute and chronic pancreatitis (Singh and Simsek, 1990). Nevertheless, controversy still exists regarding the connection between alcohol consumption and pancreatitis. The mechanisms by which ethanol either causes chronic pancreatitis itself or sensitize the pancreas to injury by other factors are not well understood. The pathology of chronic alcoholic pancreatitis presents as slowly progressive fibrosis and acinar atrophy in the obstructed pancreatic lobule consisting of both intralobular pancreatic acinar fibrosis and interlobular pancreatic duct fibrosis (De Angelis et al., 1992). The damage to the pancreas by alcohol is thought to be caused by different effects of ethanol and its metabolites.

Indeed, free radicals as byproducts of ethanol metabolism are involved in alcohol-induced tissue damage (Knecht et al., 1995). First, the alcohol non-oxidative pathway metabolism generates a large amount of fatty acid ethyl ester (FAEE) synthase that catalyzes the formation of FAEEs. In vitro studies of isolated acinar cells have confirmed that FAEEs cause acinar cell ATP depletion that affects intracellular (e.g., ER) calcium stores, resulting in the increase in cytosolic Ca^{2+}. The subsequent mitochondria overload leads to the inability to produce ATP. The end result is acinar cell death (Gukovskaya et al., 2002). Besides, alcohol is also metabolized by the oxidative enzyme system using alcohol dehydrogenase or the cytochrome P450 system. Reactive oxygen species are generated during cytochrome metabolism, initiating tissue injury via the activation of the NF-κB pathway and increased transcription rates of pro-inflammatory cytokines (Pandol et al., 1999). In addition, ethanol is dehydrogenated to aldehyde, forming a variety of damaging free radicals and reactive oxygen species (Pandol et al., 1999). Finally, ROS are also generated through NADPH oxidase from the infiltration of activated inflammatory cells. Notably, the absence of necessary ethanol metabolism-associated enzymes in the pancreas may even worsen the damage because of the accumulation of toxic

byproducts.

The pancreatitis model induced by caerulein (a cck analog derived from the Australian tree frog *Litoracaerulea*) is widely used as one of the best characterized experimental types ever since 1977 (Lampel and Kern, 1977). As an analog of cholecystokinin (CCK), caerulein causes the dysregulation of the production and secretion of digestive enzymes of the pancreas, which affects the maximum pancreatic secretion of amylase and lipase (Jensen et al., 1989; Shalbueva et al., 2013), and leads to cytoplasmic vacuolization, acinar cell death, and the infiltration of inflammatory cells into the pancreas (Lerch and Adler, 1994; Willemer et al., 1992). Notably, during the initiation and the development of caerulein pancreatitis, ROS play a role as important mediators (Aho et al., 1982; Okumura et al., 1982). Once largely produced by the NADPH oxidase from various dysregulated pancreatic cells (mainly stimulated neutrophils) in pancreatitis, ROS contribute to a large amount of cytokine production in acinar cells by directly activating the oxidant-sensitive transcription factor, NF-κB. ROS production may also disturb the integrity of the cytoskeleton and induce mitochondrial dysfunction (Crimi et al., 2007; Snook et al., 2008). By disturbing the activation of various cytokines, ROS also take part in mediating the activation of the Jak/Stat pathway. The latter is believed to mediate a wide variety of biological effects such as the immune response, differentiation, cell survival, proliferation and oncogenesis (Rawlings et al., 2004). In addition, ROS are important mediators of apoptosis (Sandoval et al., 1996). In caerulein-induced pancreatitis, a high degree of ROS is also found to be associated with the promoted expression of proapoptotic gene bax and p53 as well as DNA fragmentation, suggesting that the p53/caspase pathway is also involved in caerulein pancreatitis (Yu et al., 2003; Yu et al., 2005).

1.3.2. Types of Alcoholic chronic pancreatitis mouse models

Since the 1960s when Lieber and DeCarli performed a series of studies investigating the effects of alcohol on the liver, various animal models have been developed that focus on the effects of alcohol or other stimuli on the pancreas (Lieber et al., 1965). Although a variety of animal models for chronic pancreatitis exist, the goal of developing a good animal model has not been achieved, and the currently available models are clearly imperfect. Here, we propose that an ideal chronic pancreatitis model includes the following characteristic. First, the model should be mainly ethanol dependent to replicate the clinical condition because chronic pancreatitis is the most common result of long term ethanol abuse. Second, an in vivo model should allow for studies to replicate the body environment for a better understanding of the mechanisms regulating this condition. Third, the model should use mice to take advantage of the genetic mouse models that are currently available for mechanistic studies. Here, we compared several widely used mouse models.

1.3.2.1. The intragastric ethanol infusion model (Tsukamoto et al., 2008)

- Mice: 8-10 weeks
- Operated under general anesthesia for implantation of a long-term gastrostomy catheter
- 1 week of a controlled high-fat diet
- Ethanol infusion was initiated at a dose of 18g/kg/d, increased by 1.5g every 2 days until it reached 29g/kg/d
- Advantage: The most common paradigms and more controlled which allows accurate control of nutrient intake and alcohol consumption patterns
- Disadvantage: expensive, complicated, and the living quality of the mice is low

1.3.2.2. The "Lieber-DeCarli Formula" method (Kono et al., 2001)

- Mice: 8-10 weeks old

- Fed with Lieber-DeCarli liquid-based diet for 6-8 weeks
- Fed with Lieber-DeCarli liquid-based diet containing Lieber-DeCarli diet or the for eight weeks
- Collect tissue
- Advantage: Easy to access
- Disadvantage: A less controlled approach; the consuming of the liquid diet should be calculated every day

1.3.2.3. The "Lieber-DeCarli Formula" method plus caerulein injections (Kamst et al., 1995)

- Mice: 8-10 weeks old
- Fed with either the non-alcohol containing Lieber-DeCarli diet or the Lieber-DeCarli diet containing 24% of calories as ethanol
- After eight weeks mice were given seven hourly intraperitoneal injections of a stimulating dose of caerulein on three alternate days of three consecutive weeks
- Tissue were collected after the last injection of caerulein
- Advantage: Easy to access
- Disadvantage: A less controlled approach

1.3.2.4. The ethanol injection plus caerulein (Charrier and Brigstock, 2010)

- Mice 8-10 weeks old fed with a low-fat diet
- Injected with ethanol (3.2 g/kg; administered in a 33.3% ethanol: 67.7% water solution)
- One time per day, six times per week, for three weeks
- On one day each week, some mice also received an i.p. injection of caerulein every hour for six hours. (50 µg/kg)
- Control mice received either ethanol alone, cerulean alone, or water alone
- Advantage: Administration of ethanol and cerulean three times a week

together to mice results in profound pancreatic fibrosis; The short time frame of protocol substantially reduces animal housing costs; Ethanol intake can be exquisitely controlled by simple i.p. injection

- Disadvantage: Short time protocol might not perfectly simulate the ACP model

To sum up, we suggest that last murine model should be ideally suited for studies aimed at elucidating the pathophysiological and pathogenetic mechanisms underlying the development of ethanol induced chronic pancreatitis only with minor modification.

2. AIMS OF THIS STUDY

A better understanding of the early mechanisms that lead to PDAC may identify novel molecular targets for the early diagnosis or prevention of PDAC.

During the past decades, few studies have focused on the role of inflammation/ROS in the early stage of PDAC carcinogenesis. Most studies aimed to identify genetic changes and the role of these alterations in pancreatic cancer development. However, recently, more studies have shown that inflammation together with ROS play a role in the early stages of pancreatic carcinogenesis (Goto et al., 1991; Greer and Whitcomb, 2009; Whitcomb, 2004). However, the underlying mechanism is not fully understood. A valid inflammation/ROS generation model is required to mimic in vivo conditions before tumor formation. Here, we aim to establish in vivo ROS generation model.

As TSC-mTOR pathway may performs a largely underestimated role in pancreatic carcinogenesis. The role of TSC1-TSC2 in the development of PDAC needs to be defined. In addition, since p53 inactivates mTORC1 together with TSC function through a negative feedback signal loop, the dysfunction to one or two points in the loop may imbalance the feedback mechanism, leading to an unpredictable cell fate. What are the outcomes of high levels of ROS generation in the impaired loop? Therefore, the biological significance of the signal loop in the pancreas needs to be analyzed.

To this end, the following 2 major hypotheses have been formed.

Hypothesis 1: The inflammation level corresponds to the amount of ROS generation

Hypothesis 2: The transcription factor p53 plays an essential role in ROS generation by interacting with the Tsc/mTORC1/p53 signal loop pathway

To address these hypotheses, the following questions have been specifically asked.

Question 1 (Q1): Is ROS upstream of inflammation or a downstream target of inflammation? How is inflammation affected if ROS production is inhibited?

Question 2 (Q2): How does p53 regulate and control the ROS level?

Question 3 (Q3): Does apoptosis affect the tissue inflammation level?

Question 4 (Q4): How is ROS or inflammation affected if the Tsc/mTORC1/p53 pathway is impaired?

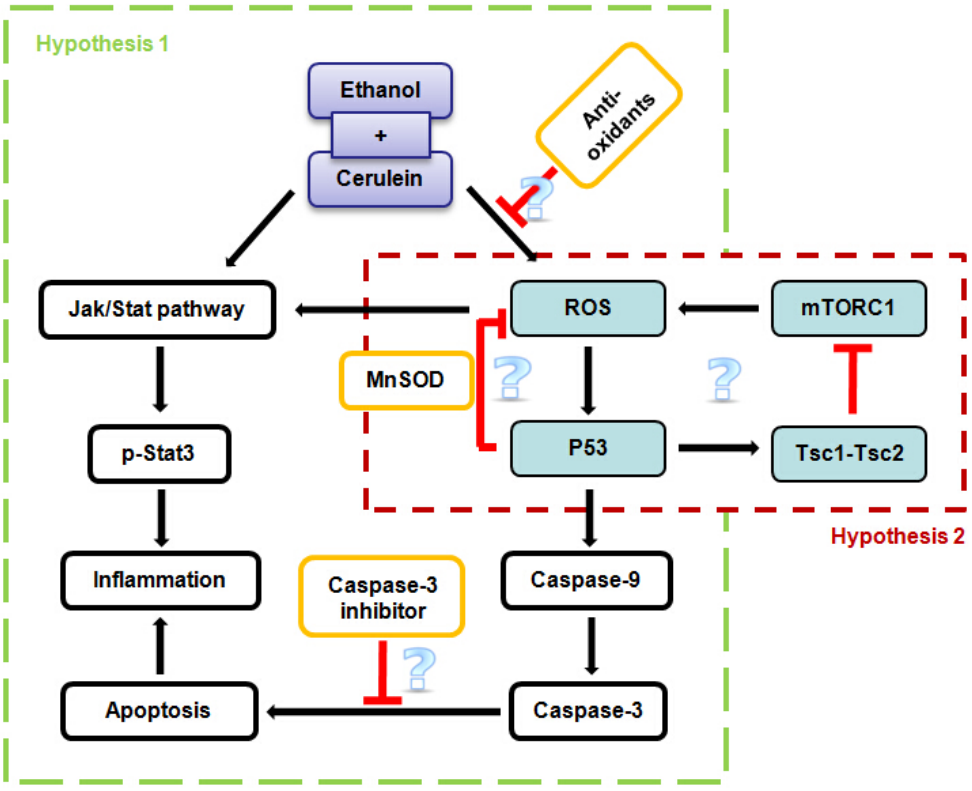

Figure 4: Schematic figure illustrating the structure of this study

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals and Reagents

0.25% trypsin/EDTA	Invitrogen (Carlsbad, CA, USA)
2-Mercaptoethanol	Sigma-Aldrich (St. Louis, MO, USA)
Acetic acid	Merck Biosciences (Darmstadt, Germany)
Acetic anhydride	Sigma-Aldrich
Acrylamide solution	ROTH (Karlsruhe, Germany)
Agarose	ROTH
Ampicillin	Sigma-Aldrich
Ammonium persulfate (APS)	Sigma-Aldrich
Biocoatmatrigel invasion unit	BD Biosciences (Franklin Lakes, NJ, USA)
Bovine serum albumin (BSA)	ROTH
Bromophenol blue	Sigma-Aldrich
Calcium chloride	Merck Biosciences
Caerulein	Sigma Aldrich
Chloroform	Merck Biosciences
Crystal violet	Merck Biosciences
Dextran	Merck Biosciences
Dextran sulfate	Sigma-Aldrich
Dimethyl sulfoxide	Sigma-Aldrich
Dinatriumhydrogenphosphate	Merck Biosciences
ECL detection reagent	Amersham (Little Chalfont, UK)
Eosin	Sigma-Aldrich
Ethanol	ROTH
Ethidium bromide	ROTH
Fetal calf serum	PAN Biotech (Aidenbach, Germany)
Formamide	Merck Biosciences
Gelatine	ROTH
Glycerol	Merck Biosciences
Glycine	ROTH
Haematoxylin	Merck Biosciences
Hydrogen peroxide (30%)	ROTH
Histowax	Leica (Wetzlar, Germany)
Humidified chamber	TeleChem (Sunnyvale, CA, USA)
Isfoluran	CP-Pharma, Burgdorf
Isopropyl b-D-thiogalactopyranoside	Sigma-Aldrich
Isopropanol	ROTH
Potassium dihydrogen phosphate	Merck Biosciences
Laurylsulfate (SDS)	Sigma-Aldrich
Liquid nitrogen	Tec-Lab (Taunusstein, Germany)

Liquid DAB &chromogen substrate	DakoCytomation
Methanol	Merck Biosciences
Milk powder blotting grade	ROTH
Molecular weight marker	FERMENTAS/Termo (Waltham, USA)
Normal goat serum	DakoCytomation
Nitrocellulose membranes	Bio-Rad (Hercules, CA, USA)
Para-formaldehyde	Apotheke TU München (Munich, Germany)
PBS powder without Ca^{2+}, Mg^{2+}	Biochrom AG (Berlin, Germany)
Phosphate buffered saline (PBS) pH 7.4	Invitrogen
Polyvinylpyrrolidone	Sigma-Aldrich
Potassium chloride (KCl)	Merck Biosciences
Permount	Vector Laboratories (Burlingame, CA, USA)
Protease inhibitor cocktail	Roche diagnostics (Penzberg, Germany)
Proteinase K	Sigma-Aldrich
RNAseDNAse-free water	Invitrogen
Roticlear	ROTH
Sodium borate	Merck Biosciences
Sodium chloride	Merck Biosciences
Sodium citrate	Merck Biosciences
Sodium phosphate	Merck Biosciences
TEMED	Sigma-Aldrich
Toluidine blue	Merck Biosciences
Triethanolamine	Sigma-Aldrich
Tris base	Merck Biosciences
Tween 20	Merck Bioscience
Z-DEVD-FMK (Caspase-3 inhibitor)	Santa Cruz Biotechnology

3.1.2.Size and mass standards

GeneRuler 1kB DNA ladder	Fermentas, Schwerte
PageRulerprestained protein ladder	Thermo Scientific

3.1.3.Enzymes

Proteinase K	Peqlab, Erlangen
Proteinase inhibitor	Peqlab
Phosphatase inhibitor	Peqlab

3.1.4.Special diet

Butylated Hydroxyanisole (BHA) contained food	Research Diets, Inc (New Brunswick, USA)

3.1.5. Kits

BCA protein assay kit	Thermo Scientific
QIAquick purification kit	Qiagen (Hilden, Germany)

QuantiTect Rev. Transcription Kit	Qiagen
RNeasy Plus Mini Kit	Qiagen
QIAquick Gel Extraction Kit	Qiagen
QIAGEN DNA Mini Kit	Qiagen
SYBR Green 1 Master kit	Roche diagnostics
OxiSelect TBARS Assay Kit	Cell Biolabs.Inc (San Diego, CA, USA)

3.1.6. primers

3.1.2.1. Sequences of primers used for QRT-PCR analysis of the mouse genes:

Gene Name	Sense (5'→3')	Antisense (5'→3')
SOD1	AACCAGTTGTGTTGTCAGGAC	CCACCATGTTTCTTAGAGTGAGG
SOD2	CAGACCTGCCTTACGACTATGG	CTCGGTGGCGTTGAGATTGTT
SOD3	CCTTCTTGTTCTACGGCTTGC	TCGCCTATCTTCTCAACCAGG
GPX1	AGTCCACCGTGTATGCCTTCT	GAGACGCGACATTCTCAATGA
GPX2	GCCTCAAGTATGTCCGACCTG	GGAGAACGGGTCATCATAAGGG
GPX3	CCTTTTAAGCAGTATGCAGGCA	CAAGCCAAATGGCCCAAGTT
GPX7	TCCGAGCAGGACTTCTACGAC	TCTCCCTGTTGGTGTCTGGTT
PpiB	GGAGCGCAATATGAAGGTGC	CTTATCGTTGGCCACGGAGG

3.1.7. Sequences of primers used for genotype:

Gene Name	Sense (5'→3')	Antisense (5'→3')
$P48^{cre}$	ACTCGGATGCCGACACGGGA	CCTGGCCTTGCTTGCTCCCC
TSC1	TCCTGGCTACTCTGGAGACC	TTCCATGGTGTAATGGCCCC
P53	GATGCCGACTTGGACATTGC	CACACTCCAGTTTGGCTCCT

3.1.8. List of antibodies

Primary antibodies

Antibody name	Catalog number	Application* (Reactivity**)	Producer
Rabbit Anti-p-mTORer2448 mAb#	5536	WB (M)	Cell Signaling Technology (NEB, Frankfurt/Main,Germany)
Rabbit Anti-p-mTORSer2448 mAb#	2976	IHC (H, M)	Cell Signaling Technology
Rabbit Anti-mTORmAb#	2983	WB; IHC (M)	Cell Signaling Technology
Rabbit Anti-S6 mAb#	2217	WB (M)	Cell Signaling Technology
Rabbit Anti-Cleaved Caspase3 mAb#	9664	WB; IHC (M)	Cell Signaling Technology
Rabbit Anti-Tsc1 mAb#	6935	WB (M)	Cell Signaling Technology

Rabbit Anti-p-stat3 mAb#	9145	WB; IHC	Cell Signaling Technology
Rat Anti-CD45mAb#	550539	IHC9(M)	BD Bioscience(Heidelberg, Germay)
Rabbit Anti-MalondialdehydemAb#	Ab 6463	IHC (M)	Abcam (Cambridge, UK)
Rabbit Anti-VimentinmAb#	ab92547	IHC, IF (M)	Abcam
Mouse Anti-8-Hydroxyguanosine [N45.1] mAb#	Ab48508	IHC (M)	Abcam
Mouse Anti-α-SMA mAb#	M0851	IHC (M)	Dako Deutschland GmbH (Hamburg, Germany)
Rabbit Anti-GAPDH Ab#	sc-25778	WB (H, M)	Santa cruz biotechnology (Heidelberg, Germany)
Mouse Anti-β actin Ab#	sc-69879	WB (H, M)	Santa cruz biotechnology
Rabbit Anti P53 Ab#	IHC-00053	WB(H, M) IHC (H, M)	Leica, Wetzlar

Secondary antibodies

Antibody name	Catalog number	Application*	Producer
Rabbit HRP (horseradish peroxidase)- labelled Anti-Rat IgG Ab#	P0450	IHC	Dako Deutschland GmbH (Hamburg, Germany)
Goat HRP-Labelled Polymer Anti-Mouse Ab#	K4001	IHC	Dako Deutschland GmbH
Goat HRP-Labelled Polymer Anti-Rabbit Ab#	K4003	IHC	Dako Deutschland GmbH
Goat Alexa Fluor 488 Anti-Mouse IgG Ab#	115-546-062	IF	Dianova (Hamburg, Germany)
Chicken Alexa Fluor 594 Anti-Rabbit IgG Ab#	A-21442	IF	Invitrogen (Carlsbad, CA, USA)
Sheep HRP-labelled Anti-Mouse IgG Ab#	NA931	WB	GE Healthcare (Little Chalfont, UK)
Donkey HRP-labelled Anti-Rabbit IgG Ab#	NA934	WB	GE Healthcare

*Application key: WB= western-blot; IHC= Immunohistochemistry; IF = Immunofluorescence; **Reactivity key: H = human; M = mouse; Ab#: antibody

3.1.9. Laboratory equipment

Analytic balance	METTLER (Giessen, Germany)
Balance	SCALTEC (Göttingen, Germany)
Biophotometer	Eppendorf (Hamburg, Germany)
Centrifuge	Eppendorf
CO_2 incubator	SANYO (Secausus, NJ, USA)
Computer Hardware	Fujitsu SIEMENS (Tokyo, Japan)
Electrophresis/Electroblotting equipment/ power supply	Invitrogen
Freezer -20°C	LIEBHERR (Bulle, Switzerland)
Freezer -80°C	Heraeus (Hanau, Germany)
Lightcycler 480	Roche diagnostics (Penzberg, Germany)
Microplate Reader	Thermo Scientific
Microscope	Leica (Wetzlar, Germany)
Microwave oven	SIEMENS (Munich, Germany)
Nanodrop	Thermo Scientific
PH-meter	BECKMAN (Washington, D. C., USA)
Power supply	BIOMETRA (Göttingen, Germany)
Refrigerator 4°C	COMFORT (Buller, Switzerland)
Roller mixer	STUART (Stone, UK)
Scanner	Canon (Tokyo, Japan)
Sterilgard Hood	Thermo Scientific
Thermomixer	Eppendorf
Vortex Mixer	NEOLAB (Heidelberg, Germany)
Water bath	LAUDA (Lauda-Koenigshofen, Germany)
Tissue embedding machine	Leica (Wetzlar, Germany)
Tissue processor	Leica
Vacuum tissue processor ASP200s	Leica

3.1.10. Consumables

Cell scraper	SARSTEDT (Nuembrecht, Germany)
Coverslips	MENZEL (Braunschweig, Germany)
Filter (0.2µm)	NEOLAB (Heidelberg, Germany)
Hyperfilm	GE Healthcare
Pure Nitrocellulose membrane (0.45µM)	BIO-RAD
Sterile needles	BD (Franklin Lakes, NJ, USA)
Tissue culture dishes (60x15mm; 100x20mm)	GREINER (Frickenhausen, Germany)
Tissue culture flasks (25cm^2; 75 cm^2; 125 cm^2)	GREINER
Tissue culture plates (6-well; 24-well; 96-well)	GREINER

Tubes (15ml; 50ml)	GREINER
Blotting paper	Whatman (Maidstone, Kent, UK)

3.2. Methods

3.2.1. DNA isolation for genotyping

Mice were anaesthetized with isofluran. Afterwards pieces of tail were dissected for further treatment. Tissue was incubated in 500µlSTE buffer and 15 µl proteinase K (20mg/ml to a total concentration of 1mg/ml) for tail samples. Then the tubes were put over night at 55°Cand 550rpm TS1 thermoshaker (Biomwtra, Jena, Germany).

After it was centrifuged at 130000 g for ten minutes, the supernatant was transferred into a new tube and 400µl of isopropanol were added to each probe to precipitate the DNA. After ten minutes of room temperature incubation, the supernatant was centrifuged another ten minutes at 13000 g. The supernatant was discarded and the remaining DNA pellet was washed with 70% ethanol. The tube was dried before resuspension of the DNA. 50µl DNase-free water was added. The DNA was stored at -20°C till usage.

STE Buffer

NaCL	0.1M
Tris-Hcl	10mM
EDTA	1mMf
SDS	1%
pH	8
Aqua dest	ad

3.2.2. RNA isolation

For RNA isolation of tissue samples, small pieces (0.5cm^3) were homogenized in 500µlRNAlaterreagent. Samples were then centrifuged at full speed for 15 minutes and the clear supernatant was transferred to RNeasyPlus Mini Kit (Qiagen, Hilden, Germany) spin columns. The isolation was carried out according to the manufacturer's instructions. After isolation the RNA samples were resolved

in 30µl of RNase-free water. The RNA was store at -80°C till usage.

3.2.3. Evaluation of RNA quality

The concentration of the freshly isolated mRNA is tested by the nanodrop (Thermo scientific, Wilmington, USA).1µl RNA was needed for the evaluation. The most important parameter concerning the quality of RNA is the absorbance proportion at wavelength 260nm and 280nm. A ratio of ~2.0 was recommended as pure RNA.

3.2.4. cDNA synthesis

For cDNA synthesis 1µg RNA was reverse transcribed with QuantiTect Rev. Transcription Kit. The reaction was carried out according to the manufacturer's instructions. The final cDNA concentration was adjusted to 20ng/µl with ddH_2O and then stored at -20°C for further use.

3.2.5. Polymerase Chain Reaction RCR (genotyping, primertest)

The PCR served as an amplification step of specific DNA fragments. 1µl ddH_2O contain 20ng cDNA was used for the amplification together with 12.5µl of mastermix, 10.5µl ddH_2O and 1µl of specific primer (10nM). The procedure took place in a TermocyclerDoppio (VWR, Ismaning, Germany). Denaturation was performed at 94°C, the annealing temperature was 58°C and elongation at 72°C. A total of 40 cycles were set up to complete to program. The final products were separated by length with a 2%agarose gel. A gel-documentation-chamber (VWR, Ismaning, Germany) was used to detect the DNA fragments via ultra violet radiation.

3.2.6. Quantitative Real Time PCR (QRT-PCR)

Quantitative real time PCR (QRT-PCR) was carried out using the LightCyclerTM480 system with the SYBR Green 1 Master kit (Roche diagnostics, Penzberg, Germany). Expression of the target gene was normalized to the mouse housekeeping gene *PpiB* using the LightCyclerTM480 software release 1.5,

version 1.05.0.39 (Roche diagnostics, Penzberg, Germany). The cycle threshold (CT) value describes the cycle of the PCR in which the fluorescence signal gets significant.

3.2.7. Immunohistochemistry (IHC) analysis

3.2.7.1. HE staining

Consecutive paraffin-embedded tissue sections (2.5µm thick) were deparaffinized with Roticlear for ten minutes for three times and a descending alcohol row (two minutes each). Afterwards the section was washed in the tap water and then was put into haemalaun solution for 30 seconds (Merck, Darmstadt, Germany). After this the slice was washed with water for at least 15 minutes. Eosin was stained afterwards for 5 seconds. The slice was washed in tap water and dehydrated by an ascending alcohol row and a final immersion in Roticlear for three times. Finally, the slice was mounted with Vectashield mounting medium (Vector Labs, Burlingame CA, USA)

3.2.7.2. IHC staining

Immunohistochemistry was performed using the Dako Envision System (DakoCytomation GmbH, Hamburg, Germany). Consecutive paraffin-embedded tissue sections (2.5µm thick) were deparaffinized with Roticlear for ten minutes for three times and a descending alcohol row (two minutes each). Antigen retrieval was performed by pretreatment of the slides in citrate buffer (pH 6.0; 10mM Citric Acid) in a microwave oven for 10 minutes. After cooling down for at least 30 minutes, endogenous peroxidase activity was quenched by incubation in menthol containing 3% hydrogen peroxide at room temperature for 10 minutes. Then the slides were blocked by nonspecific reactivity with TBS (pH 7.4; 0.1M Tris Base, 1.4M NaCl) containing 3% BSA or goat serum. The respective primary antibodies were added to the slides at 4°C overnight followed by incubation with horseradish peroxidase-linked goat anti-rabbit or mouse second antibodies. DAB color-reaction were used and followed by a counterstaining with Mayer's

hematoxylin.

3.2.8. Serum ROS level detection

Lipid peroxides are unstable indicators of oxidative stress in cells that decompose to form reactive compounds Malondialdehyde (MDA), which is relative stable by-product of lipid peroxidation.TheThiobarbituric Acid Reactive Substances (TBARS) Assay Kit is a tool for the direct quantitative measurement of MDA in biological samples. Mice serum was collected freshly from sacrificed mice and added with 100X BHT to achieve a final concentration of 1X so as to prevent further oxidation. The unknown MDA containing samples or MDA standards are first reacted with TBA at 95°C. After a brief incubation, the samples and standards can be read via either spectrophotometrically or fluorometrically. The MDA content in unknown samples was determined by comparison with the predetermined MDA standard curve. Each MDA-containing samples and standard were assayed in duplicate. The remaining serum can be stored in -80°C for further use.

3.2.8. Serum Inflammation marker detection

Freshly isolated serum from sacrifice mice was immediately diluted 1:1 with distilled water. Cobas ® 8000 (Roche diagnostics, Penzberg, Germany) was used to detect the serum Amylase, LDH, Lipase level. The remaining serum can be stored in -80°C for further use.

3.2.9. Mouse treatment

3.2.9.1 Mouse lines

Mice containing a floxed allele of Tsc1 (005680), and p53 (008462) were obtained from Jackson Laboratory (Bar Harbor, USA). The pancreas-specific Crerecombinase line $p48^{Cre/+}$ was a generous gift from PD Dr. Jens T. Siveke (Department of Internal Medicine, Technical University of Munich). The exon 17 and 18 of the Tsc1 allele and the exons 2-10 of p53 in these animals are flanked by two LoxP sites, respectively, as described previously (Kwiatkowski et al., 2002;

Marino et al., 2000). The wild type mice (*WT*) were bred in the specific pathogen free (SPF) mouse facility at the Technical University of Munich with a mixed genetic background.

3.2.9.2. Mouse breeding

Mouse breeding was performed and husbandry was maintained at the specific pathogen free (SPF) mouse facility at the Technical University of Munich. The compound transgenic mice were maintained on a mixed background. All mouse experiments and procedures were approved by the Institutional Animal Care and Use Committees of the Technical University of Munich. All procedures were in accordance with the Office of Laboratory Animal Welfare and the German Federal Animal Protection Laws.

3.2.9.3. Alcohol chronic pancreatitis mouse model

Animal model was performed to all the mouse line described above respectively according to the previous study with minor modification (Charrier and Brigstock, 2010). Different groups were designed. Briefly for the standard experiment group, randomly selective mice around 8-10 weeks were treated with both ethanol and caerulein. On day 1-6, ethanol (3.2 g/kg; administered in a 33.3% ethanol: 67.7% water solution) was injected to the mice intraperitoneally one time per day, six times per week, for three weeks. On day 7 each week, caerulein (50μg/kg dissolved in 100μl NaCl)was injected to the mice intraperitoneally for 8 times a day to generate a recurrent acute pancreatitis. Mice were euthanized 2 days after the last injection. Pancreas, liver and serum were harvested from the mice for histological and biochemical analyses. Mice for the control group would receive either ethanol alone, caerulein alone, or water and saline (n=3). Here was also a group that mice would receive food containing antioxidant (BHA) instead of normal food to further confirm the role of ROS in mediating inflammation. In order to figure out the role of apoptosis during inflammatory response, Z-DEVD-FMK (Caspase-3 inhibitor) treatment group was also included. In this group, a

pretreatment of Z-DEVD-FMK (25µg/kg, stored in 0.1µl DMSO, dissolved in 100µl NaCl) was done intraperitoneally one hour before the caerulein injection without other changes on day 7. Other group would be given the same amount of DMSO dissolved in saline and injection to the mice as an internal control. Mice were sacrificed two days after last caerulein injection (Figure 5).

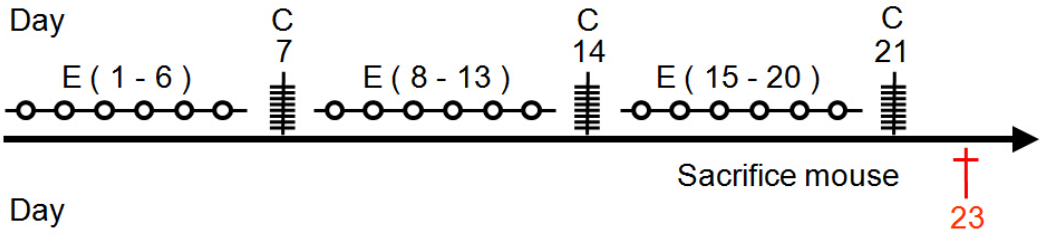

Figure 5: Temporal course of treatment and time points of tissue collection

3.2.9.4. Tissue collection

The mice were anaesthetized with isofluran and sacrificed with cervical dislocation under isofluran anesthesia. Blood was taken from Angular vein of the mice by removing the eyeball. Afterwards, serum was obtained via centrifuging the blood at 600rpm 15minutes. Pancreas and liver were dissected and were either directly frozen with liquid nitrogen or moved to fixation with 4% paraformaldehyde overnight at 4°C. Fixative tissue was kept at 4°C while frozen tissue was stored at -80°C.

3.2.9.5. Paraffin sections

Tissue was fixed in 4% paraformaldehyde 4°C over night. Afterwards samples were processed with several immersive alcohol steps in a vacuum tissue processor ASP200S(Leica,Wetzlar, Germay). Finally, the tissue was embedded in warm paraffin. After cooling down to -20°C, the blocks were cut consecutively to 2.5µm sections with a microtome(Leica, Wetzlar, Germay). The sections were then moved to slides and dried overnight at 37°C. The slides are ready to use in room temperature.

3.2.10. Statistical analysis

For statistical analyses, GraphPad Prism 5 Software (GraphPad, San Diego, CA, USA) was used. The Chi-square test or Fisher's exact test was used to compare distribution of categorical factors of different groups. All experiments were repeated at least three times. Unless otherwise stated, an unpaired Student’s t-test was used for group-wise comparisons of two groups. The level of statistical significance was set at $p<0.05$. Results are expressed as mean ± standard Error of Mean (SEM) unless indicated otherwise.

4. RESULTS

4.1. The establishment of an alcoholic chronic pancreatitis mouse model

Currently, various ethanol-based animal models (e.g., intragastric infusion or random food intake, ethanol alone or a combination of two or more stimuli) all have drawbacks, including difficult techniques, uncontrolled experimental approaches or an insignificant phenotype. With the aim of establishing a better designed ROS generation mouse model, we conducted experiments using *WT* mice with a mixed genetic background according to the protocol described by Alyssa L Charrier via intraperitoneal injection of ethanol with minor changes (Charrier and Brigstock, 2010). The procedure shortened to three weeks. Each week caerulein was used intraperitoneally to induce recurrent acute pancreatitis, which exacerbates the course of pancreatitis and enhances the fibrogenic effects in chronic pancreatitis (Jerrells et al., 2003; Perides et al., 2005). Similar to the overloaded gastric intake of alcohol, all of the mice showed physiological signs of intoxication, and major activity was restricted for at least three hours. However, all the mice returned to normal behavior. During the 3-week period, several mice gradually showed ethanol tolerance to varying degrees, and ethanol toxic behavior was resumed to one hour after the injection. The body weight of the mice was monitored without any significant changes among the groups.

4.2. Effects of ethanol, caerulein and ethanol plus caerulein on pancreas morphology

We first characterized changes in pancreatic gross pathology produced by the different treatments. We clearly observed (Fig. 6A) no gross pathological changes

in the pancreas of the standard control group (water+saline), a slightly enlarged pancreas and mild pancreas edema either in the caerulein alone or ethanol alone treatment. However, in the double treatment group, a severe inflammation phenotype was discovered, together with a pancreas and gastrointestinal tract structure disturbance.

Microscopic examination of hematoxylin-eosin stained sections revealed normal morphology in the samples obtained from the control group. Individual treatments of caerulein or ethanol alone resulted in mild pancreatitis, which was quite relevant to the gross observation, and minor pancreatic fibrosis was discovered in ethanol treatment group. In contrast, in the mice that received both ethanol and caerulein, severe pancreatitis was observed as characterized by loss of acinar cells and inflammatory infiltration, with several chronic pancreatitis features, including parenchymal fibrosis and the accumulation of stromal elements. However, these features did not occur uniformly throughout each tissue section, with several areas retaining their normal structure (Fig. 6B).

Despite the morphological changes in the pancreas, both the gross and micro changes in the liver of the mice among all these groups were thoroughly examined. Livers taken from all these groups were histologically normal, indicating that the treatment does not cause significant damage to the liver (Fig. 6C).

Overall, based on the pathology results, we propose that long sustained ethanol treatment plus recurrent acute pancreatitis accelerates the development of pancreatic fibrosis, which may indicate increased ROS generation. Thus, the double treatment group and the standard control were further examined in the subsequent experiment.

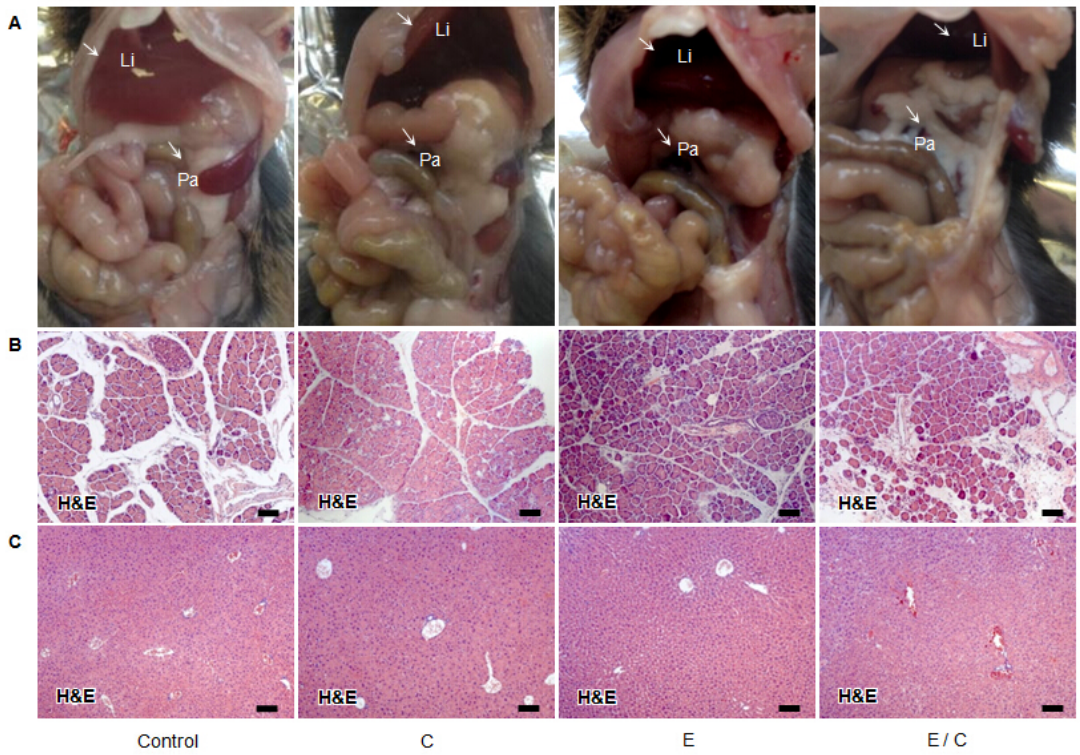

Figure 6: (n=3, scale bar: 50μm). A:Representative gross pathology of *WT* mice in four different treatment groups. B: H&E–staining of *WT* mice in different treatment groups. C: H&E–staining of the liver of *WT* mice in different treatment groups.

4.3. Effects of ethanol plus caerulein on pancreas biology

4.3.1. ROS and fibrosis

A persistent infiltration of immune cells leads to fibrosis in chronic pancreatitis and generates high ROS levels (Steer et al., 1995). The immunohistochemistry results from our mouse model shows that over a 3-week period, ethanol plus caerulein treatment caused the significantly high expression of malondialdehyde (MDA) and 7,8-dihydro-8-oxo-2'deoxyguanosine (8OHdG) (Fig. 7A, 7B). MDA is a degradation product of polyunsaturated lipids that is induced by ROS, indicating lipid peroxidation. 8OHdG is a major DNA oxidation product. Both of these

compounds are widely accepted as ROS markers. To further confirm the ROS level, the serum ROS level was also assessed using the MDA Quantitation Kit. The results also revealed an increased lipid peroxidation level in the mouse serum (Fig. 9A, 9B). Expectedly, high ROS levels are highly relevant to a strongly activated fibrosis-associated expression of α-SMA and Vimentin, which further confirms the interaction between ROS and fibrosis (Fig. 7C, 7D).

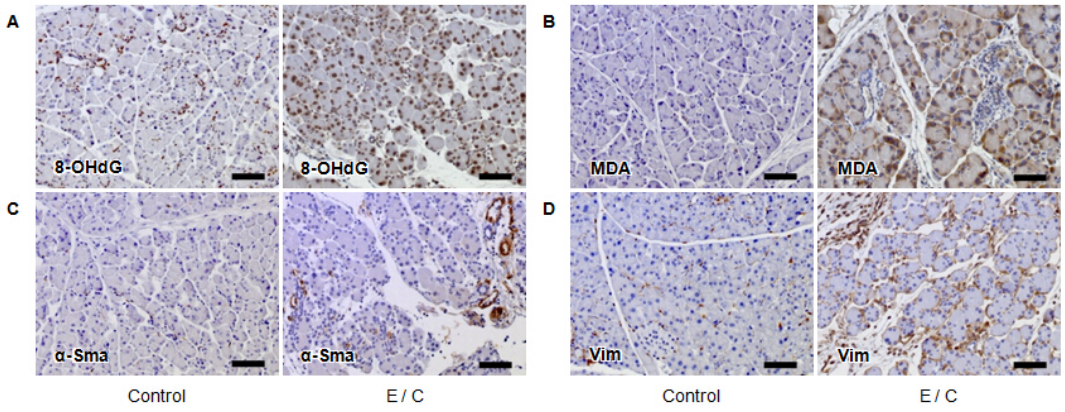

Figure 7: (n=3, scale bar: 50μm) A, B: 8-OHdG, MDA as a maker for ROS level was activated in ethanol plus caerulein group in *WT* mice. C, D: α-sma, vinmentin as stoma marker were induced in ethanol plus caerulein group *WT* mice. E-H: Representative pictures of p-Stat3, p53, cleved-caspase3 and CD45 staining for control group and double treatment group. An increased stainings of all markers in ethanol plus caerulein were discovered.

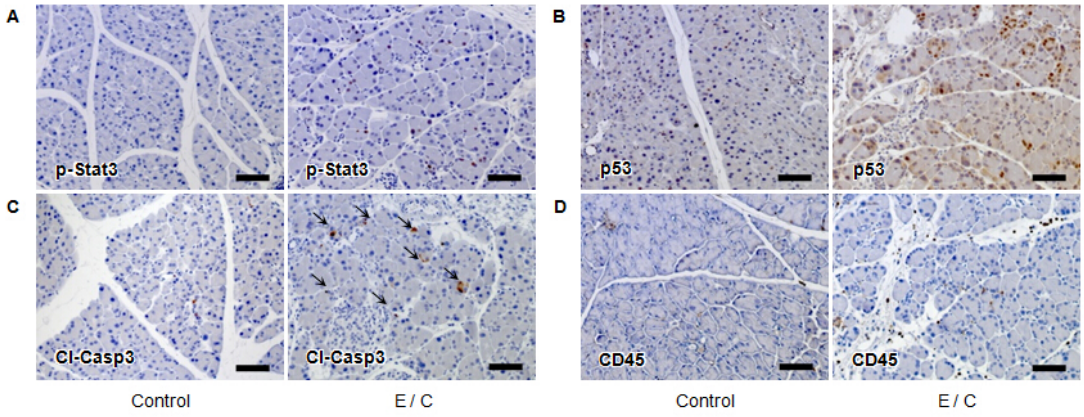

Figure 8: (n=3, scale bar: 50μm) A-D: Representative pictures of p-Stat3, p53, cleved-caspase3 and CD45 staining for control group and double treatment group. An increased stainings of all markers in ethanol plus caerulein were discovered.

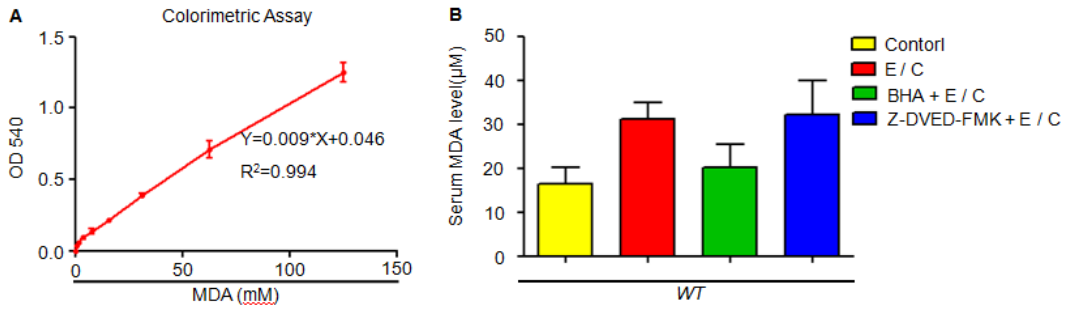

Figure 9: A: Colorimetric Assays present standard curve of the MDA experession. MDA standards were assayed in duplicate. B: The serum MDA level in ethanol plus caerulein group was significantly increased compared to water plus saline group (n=3, p<0.05), while the MDA level in mice ate with BHA contained food was significantly decreased compared to normal food group (n=3, p<0.05). Caspase-3 inhibitor did not affect the serum MDA level.

4.3.2. ROS and inflammation

Previous studies have revealed that high ROS levels are associated with the promotion of p53 expression, suggesting that p53/caspase pathway may also be involved in pancreatitis (Yu et al., 2003; Yu et al., 2005). By disturbing the activation of various cytokines, ROS also mediates the activation of the Jak/Stat pathway (Rawlings et al., 2004). Thus, we propose that ROS, inflammation and apoptosis may be highly interrelated. The IHC results were highly consistent with our hypothesis. As shown in (Fig. 8A-C), after treatment with ethanol plus caerulein, the expression of p-Stat3, p53, and cleaved-caspase-3 was significantly increased, and the ROS level was extremely higher than in the control group. CD45 staining, which indicates the infiltration of immune cells during inflammation, also increased significantly in the double treatment group (Fig. 8D). To further confirm our hypothesis, serum inflammation markers such as amylase, lipase and LDH were confirmed by cobas® 8000. Furthermore, significantly higher amylase, lipase and LDH levels were discovered in the ethanol plus caerulein group, indicating a higher inflammation level (Fig. 10A-C).

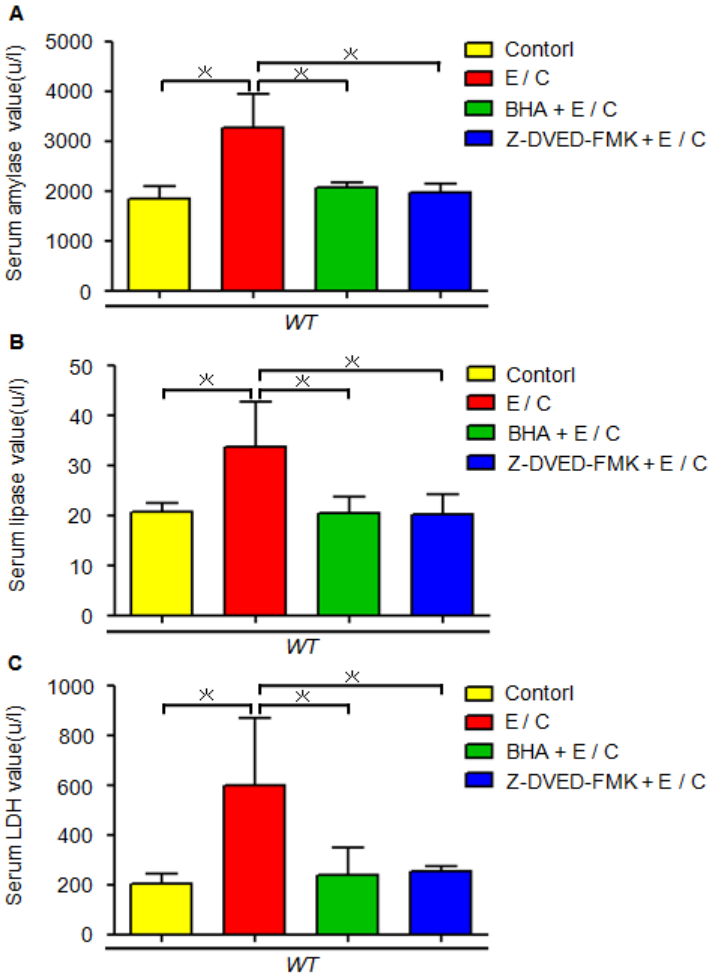

Figure 10: A-C: Representative Bar figure of amylase, lipase and LDHvalue in different treatment. (n=3, * *p<0.05*)

Overall, these data demonstrate that ROS are highly generated during the 3 week ethanol plus caerulein treatment. The inflammation level may be connected with the ROS level. p-Stat3 was activated during the treatment, which indicates that ROS may mediate inflammation through Jak/Stat pathway activation. The activation of p53 together with subsequent increases of cleaved-caspase-3 staining indicates that apoptosis may also be involved in regulating ROS or inflammation levels. However, further experiments are needed to confirm our hypothesis.

4.4. Rescue of inflammation

Here, based on all the above results, we propose that high ROS levels may

contribute to the inflammatory response of the cell via both the activation of Jak/Stat pathway and p53-dependent apoptosis. The infiltration of the immune cell caused by apoptosis mediated by p53 may, to some extent, contribute to the inflammatory response.

4.4.1. Antioxidant treatment decreases the inflammation level

Butylated hydroxyanisole (BHA) is a widely known antioxidant primarily used as an antioxidant and preservative in food, food packaging, animal feed, and cosmetics (Lam et al., 1979). Unique food containing BHA was designed in our research and was used to feed the mice (Research Diets, Inc., USA). The mice were fed with BHA-containing food ad libitum, and the other treatments described above remained the same.

After 3-weeks of treatment, changes in pancreatic gross pathology and micro morphology were compared with the mice fed normal food. Interestingly, the gross pathology presented nearly the same as the control group. The inflammatory response of the “ROS-FREE” mice was greatly weakened (Fig. 11A), and the same observations were also observed from the micro morphology experiments (Fig. 11B).

Although only a slight decrease in the ROS IHC results was observed (Fig. 12A, 12B), a significant decrease in serum lipid peroxidation was observed in the mice fed with BHA food (Fig. 9A, 9B). In addition, several important p53-related enzymes that are members of the MnSOD family such as SOD1, SOD3, GPX1 and GPX7 in the anti-oxidant system were significantly decreased in the “ROS-FREE” mice group, which indirectly revealed that BHA food successfully eliminates ROS (Fig. 15A, 15B).

Notably, ROS scavengers also appeared to affect the Jas/Stat pathway and p53-dependant apoptosis because the decrease in ROS also led to a significant decrease in the expression of p-Stat3, p53 and cleaved-caspase-3 (Fig. 13A, 13C, 13D, 14A, 14C, 14D).

Relevant to our morphological observations, the inflammation level in the “ROS-FREE” mice was greatly reduced based on the decrease in the number of CD45-positive cells (Fig. 13B, 14B). In addition, the serum amylase lipase and LDH levels also significantly decreased to approximately normal levels (Fig. 10A-C).

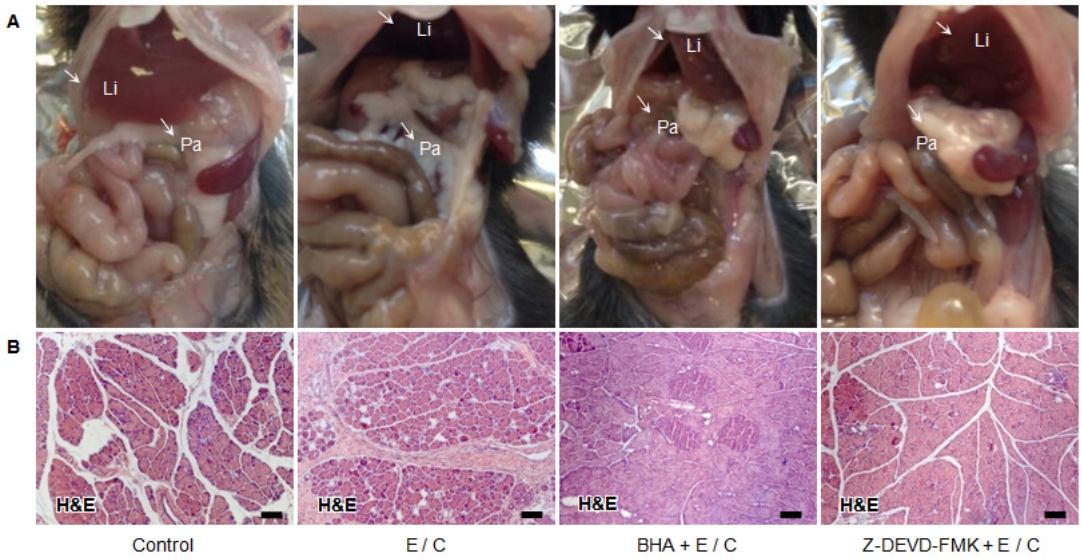

Figure11: (n=3, scale bar: 50μm). A: Representative gross pathology of *WT* mice with four different treatment groups. B: H&E-staining of *WT* mice in different treatment groups.

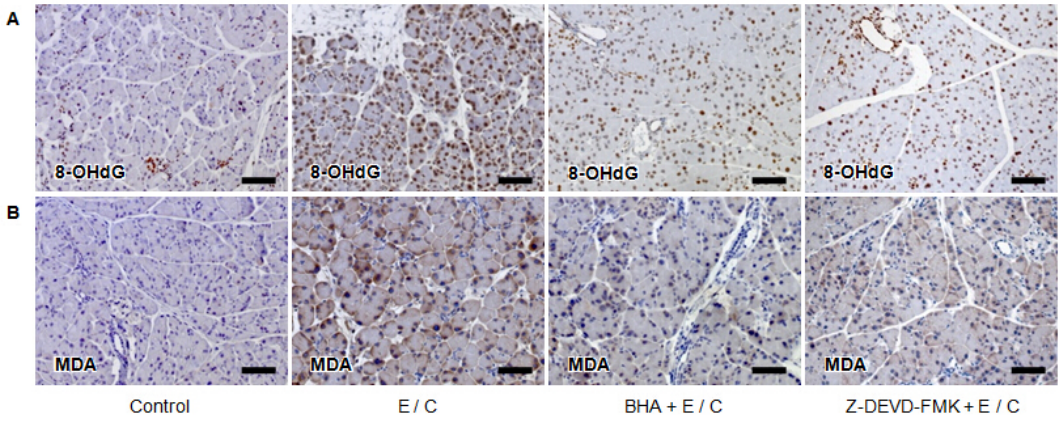

Figure12: (n=3, scale bar: 50μm). A, B: 8-OHdG level and MDA level was slightly decreased

in the mouse which ate BHA food group. Mouse pre-treated with caspase-3 inhibitor did not affect the 8-OHdG and MDA level

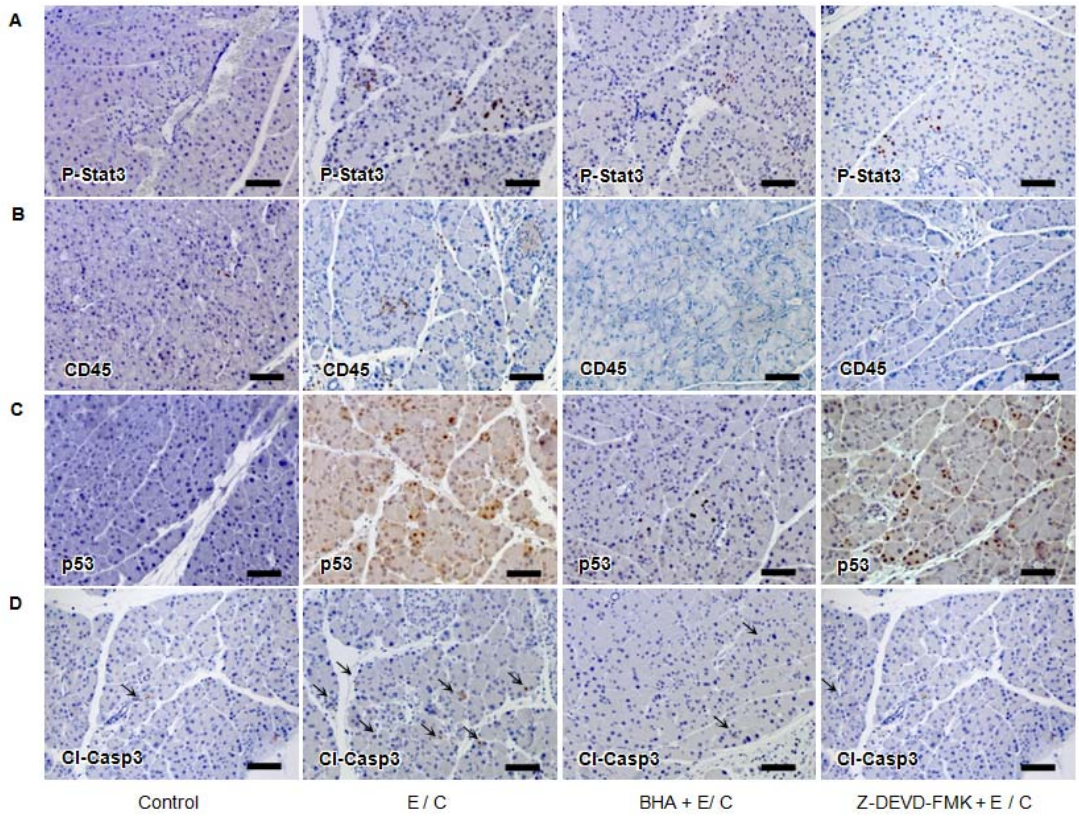

Figure 13: (n=3, scale bar: 50μm).A-D: An increased expression was discovered as described above in double treatment. Mice ate BHA containing food, showing a decreased level of p-Stat3, cleved-caspase-3, CD45 and p53 expression. No changes in the p-Stat3, p53 level in the mice pre-treated with caspase-3 inhibitor compared to control group, while cleved-caspase3 and CD45 staining was sharply reduced.

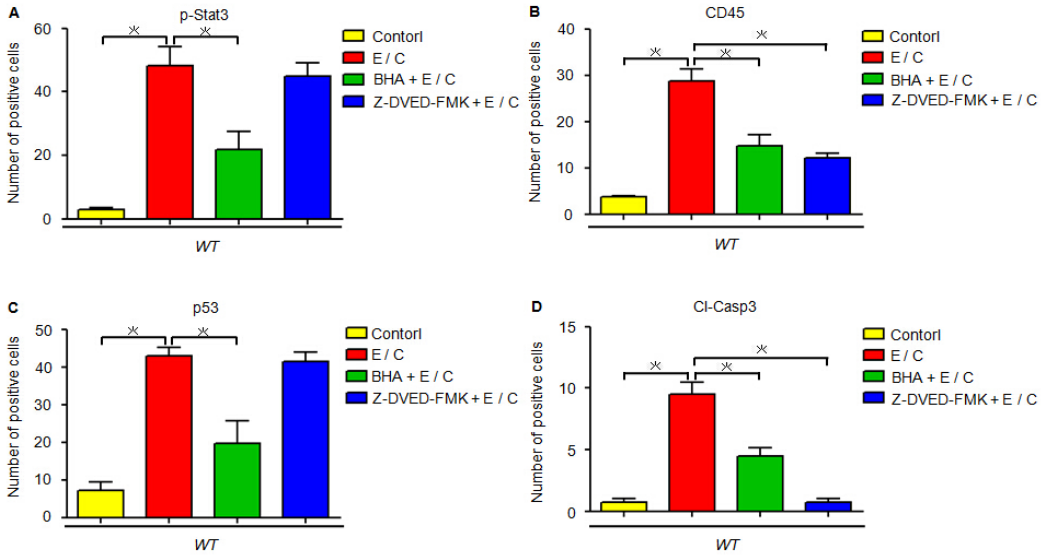

Figure14: (A-D): Statistics data of IHC results of p-Stat3, p53, cleved-caspase-3 and CD45 for each group (*$p<0.05$).

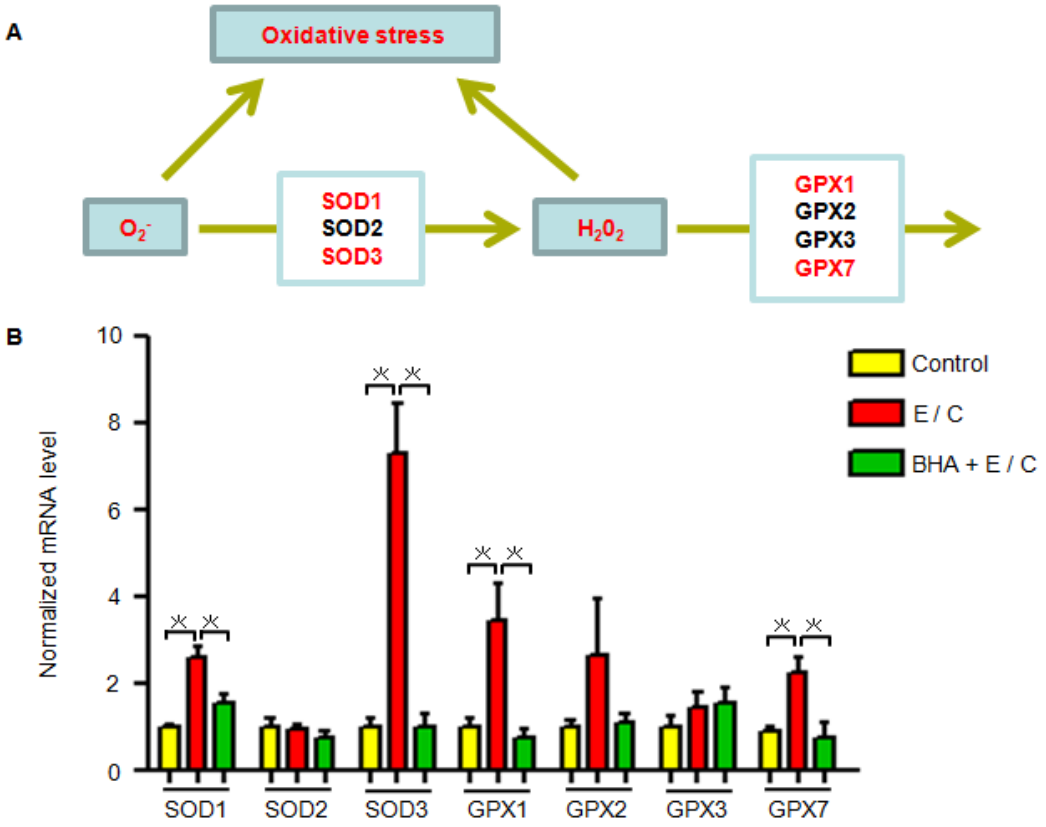

Figure 15: A: A flow chat for enzymes in anti-oxidant system. B: The relative mRNA expression of key MnSOD enzymes in different groups (*$p<0.05$).

4.4.2. Caspase-3 inhibitor reduces inflammation

To confirm the hypotheses that apoptosis also plays an important role in inflammation, Z-DEVD-FMK, a caspase-3 specific inhibitor, was used in our mice model. In total, 500ng of Z-DEVD-FMK in 100 µl of salinewas administered to the mice as previously described and presented in our methods section (Cervantes et al., 2008; Ma et al., 1998). After 3 weeks of treatment, gross and micro normal morphology were also observed compared with the ethanol plus caerulein treatment group (Fig. 11A, 11B). The barely detectable expression of cleaved-caspase-3 confirmed that caspase-3 activity was successfully inhibited in the treatment. As shown in the figure, the caspase-3 inhibitor only leads to significant decrease in the number of CD45-positive cells without affecting the expression of p-Stat3 and p53 (Fig. 13A-D,14A-D). In addition, the serum

amylase and lipase levels also significantly decreased in the caspase-3 inhibitor group (Fig. 9A-C).

Overall, these data demonstrate that the pancreas inflammation level may decrease in the absence of mediating the ROS levels. ROS, to some extent, may contribute to the inflammatory response, and p53 activation may regulate the ROS level via apoptosis and the up regulation of the anti-ROS MnSOD enzyme.

4.5. Alcoholic chronic pancreatitis mouse model using Tsc1-haploinsufficient, p53-deficient and Tsc1-haploinsufficient plus p53-deficient mice

4.5.1. Tsc1-haploinsufficiency has no significant effect on maintaining the equilibrium between ROS and inflammation

As mentioned above, mTOR signaling integrates a variety of intracellular and extracellular cellular metabolism mechanisms as well as controls mitochondrial oxidation (Cunningham et al., 2007). Up-regulated mTOR activity is associated with a higher level of intracellular reactive oxygen species (ROS) (Jang and Sharkis, 2007). More recently, Tsc has emerged as the major negative regulator of mTOR (Giaccone et al., 1990; Melnik and Schmitz, 2013). Here, we propose that impaired Tsc1 function also affects mTOR signaling, thus breaking the balance between ROS and inflammation. To investigate the role of the Tsc1-Tsc2 complex in ROS generation, we crossed *Tsc1*$^{fl/fl}$ mice with the *p48*^{Cre} line to generate transgenic *p48*^{Cre}*; Tsc1*$^{fl/+}$ (Tsc1-haploinsufficient) and *p48*^{Cre}*; Tsc1*$^{fl/fl}$ (Tsc1-deficient) mice. In these mice, one or two allele(s) of Tsc1 were specifically ablated, respectively, in the pancreas. Due to the low generation rate and high

death rate of the Tsc1-deficient mice, only Tsc1-haploinsufficient mice were treated with our standard 3-week protocol, and *WT* mice that received the same treatment were used as control. After three weeks of treatment, although histological examination of the pancreases obtained from the Tsc1-haploinsufficient mice revealed an inflammation phenotype in ethanol plus caerulein treatment compared with the water and saline group, no obvious difference was observed in the morphology in the Tsc1-deficient mice compared with the *WT* mice (Fig. 16A, 16B). ROS were also detected via the staining of 8OHdG and MDA, and as shown in (Fig. 17A, 17B), ROS were generated after double treatment. However, compared with the *WT* mice that received the same treatment, the ROS level appeared slightly lower. Unexpectedly, Tsc1 haploinsufficiency did not significantly affect mTOR signaling. The IHC results revealed that only slightly increased mTOR expression compared with the *WT* mice (Fig. 17C). While the expression of p-Stat3, p53, and cleaved-caspase-3 slightly decreased. In addition, no significant changes were discovered in the number of CD45-positive cells (Fig. 18A-D, 19A-D).

In conclusion, Tsc1 haploinsufficiency has no significant impact on mediating the balance between ROS and inflammation.

4.5.2. p53 deficiency affects mTOR signaling and may contribute to the inflammatory response mediated by ROS and inflammation

A previous study showed that p53 orchestrates mitochondrial redox signaling via the superoxide scavenger MnSOD (Pani and Galeotti, 2011). p53 activation is also reported to increase the GAP activity of Tsc1-Tsc2, which is the major negative regulator of mTOR (Budanov and Karin, 2008; Giaccone et al., 1990;

Melnik and Schmitz, 2013). To investigate the role of p53 in ROS generation, we crossed *p53*$^{fl/fl}$ mice with the *p48*^{Cre} line to generate transgenic *p48*^{Cre}*; p53*$^{fl/fl}$ (p53-deficient) mice. Two p53 allele(s) were specifically ablated in the pancreas, and morphological changes were compared between the *WT* and p53-deficient mice. An enlarged pancreas was observed in the p53-deficient mice, and regarding micro morphology, a severe acute phase phenotype and stronger immune cell infiltration were observed in the p53-deficient mice after double treatment (Fig. 16A, 16B). Notably, the ROS level in the p53-deficient mice was highly expressed and was followed by the strong expression of p-mTOR$_{s2448}$ compared with double treatment mice (Fig. 17A-C). Interestingly, p-Stat3 expression was also strongly up regulated due to loss of p53 function (Fig. 18B, 19B) CD45-positive cells were higher in number in the mutant mice compared with the *WT* mice, which was consistent with our morphological observations (Fig. 18A, 19A). In support of our other studies, the p53-deficiency disrupts the p53-dependent apoptosis pathway, which uses cleaved-caspase-3 in the final step. A fewer positive cleaved-caspase-3 cells were examined in our *p48*^{Cre}*; p53*$^{fl/fl}$ mice (Fig. 18D, 19D).

4.5.3. Effects of ethanol plus caerulein on Tsc1 haploinsufficiency and p53-deficient mice

Tsc1 deficiency hyperactivates mTORC1, which affects ROS generation in the pancreas. p53 activation regulates the function of the Tsc1-Tsc2 complex. Therefore, we assumed that p53 activity may also be involved in this process. To determine the biological relevance of p53 activation, p53 was inactivated in the context of Tsc1 haploinsufficiency and Tsc1 deficiency. *p48*^{Cre}*; p53*$^{fl/fl}$*; Tsc1*$^{fl/fl}$ and *p48*^{Cre}*; p53*$^{fl/fl}$*; Tsc1*$^{fl/+}$ mice were generated and studied. Only Tsc1-haploinsufficient and p53-deficient mice were included in our standard 3-week protocol due to the low generating rate and high early death rate of the

p48^{Cre}; p53$^{fl/fl}$; Tsc1$^{fl/fl}$ mouse line. Our previous study showed that Tsc1 haploinsufficiency does not have a significant impact on pancreatic physiology, while the inactivation of p53 appears to significantly contribute to the pancreas. Based on the gross morphology, three weeks of double treatment caused approximately no harm the pancreas tissue as the pancreas appeared normal. The micro pathology of the Tsc1-haploinsufficient and p53-deficient mice also appeared more normal compared with the *WT* mice (Fig. 16A, 16B). The Jak/Stat pathway was also weakly activated, minor apoptosis was observed, and CD45 staining was also significantly less than *WT* mice (Fig. 18A-C). Meanwhile, the disturbance of both p53 function and Tsc1 did not lead to the increased regulation of the mTOR signaling pathway. The $mTOR_{s2448}$ IHC staining was less strong than the p53-deficient group (Fig. 17C). The underlying mechanism behind this regulation requires further discussion.

In summary, three different mouse lines *p48^{Cre}; Tsc1$^{fl/+}$*, *p48^{Cre}; p53$^{fl/fl}$*, *p48^{Cre}; p53$^{fl/fl}$; Tsc1$^{fl/+}$* were generated to research the Tsc/mTORC1/p53 signal loop (Feng et al., 2005). Each signaling component was reported to be connected with the cellular ROS level. Tsc1 haploinsufficiency did not greatly affect the ROS balance and inflammation; either the inflammation level or the ROS level remained nearly the same as in *WT* mice that received the same treatment. In contrast, p53 deficiency strongly induced the mTOR level as well as high ROS generation, followed by an uncontrolled inflammatory response. However, the combination of both p53 deficiency and Tsc1 haploinsufficiency did not worsen the delicate balance of Tsc/mTORC1/p53 signal loop as expected. The findings show less inflammation and a less activated Jas/Stat pathway. Thus, we need to further discuss the mechanism underlying these observations.

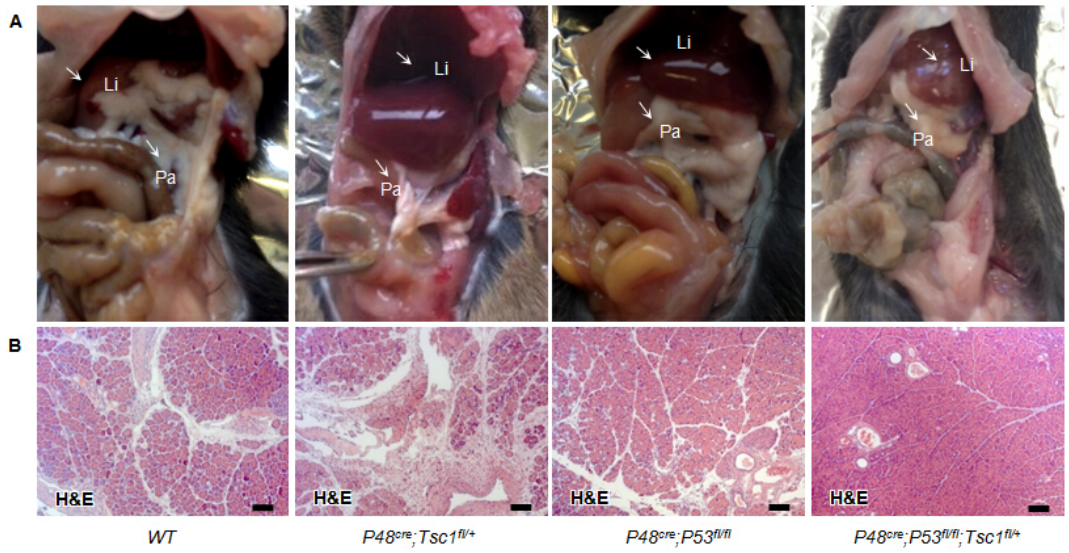

Figure 16: (n=3, scale bar: 50μm). A: Representative gross pathology transgenic mice with ethanol plus cerulean treatment. B: H&E-staining of the mice for micro morphology.

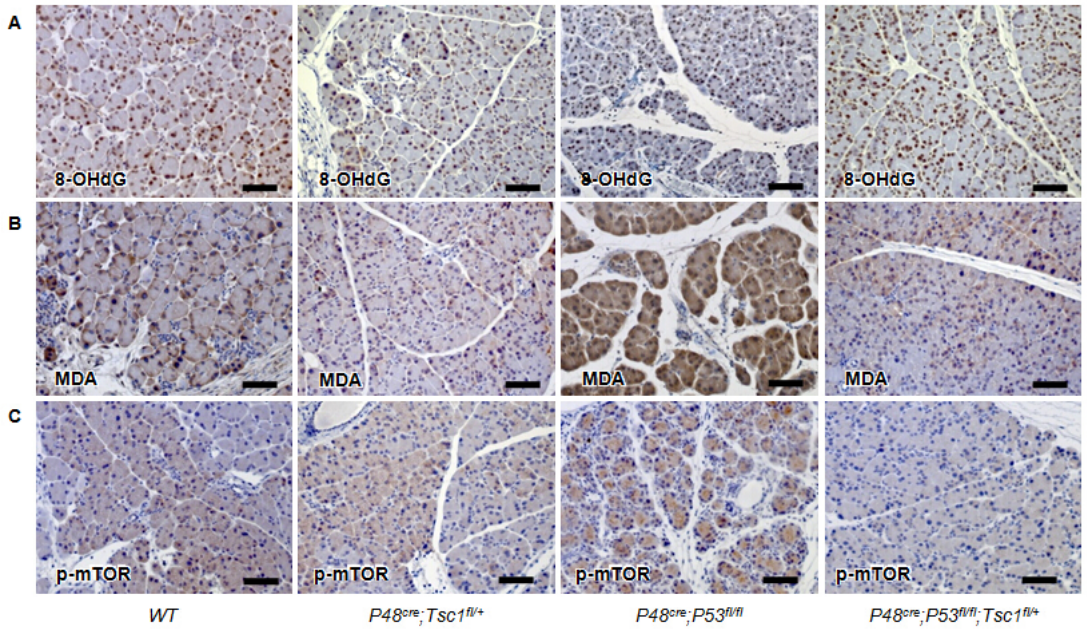

Figure 17: (n=3, scale bar: 50μm). A-C: Representative 8-OHdG, MDA and p-mTOR staining of *WT*, $p48^{Cre}$; $Tsc1^{fl/+}$, $p48^{Cre}$; $p53^{fl/fl}$, $p48^{Cre}$; $p53^{fl/fl}$; $Tsc1^{fl/+}$ mice.

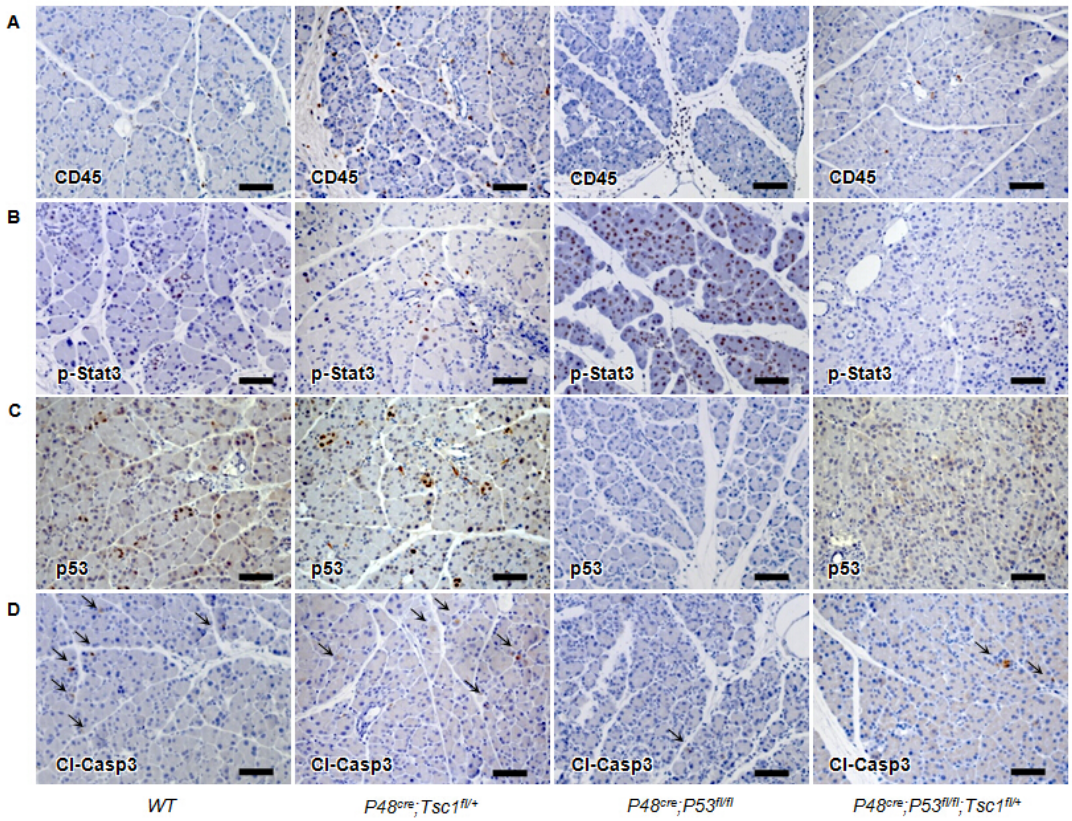

Figure 18: (n=3, scale bar: 50μm). A-D: IHC staining of *WT*, *p48^{Cre}; Tsc1$^{fl/+}$*, *p48^{Cre}; p53$^{fl/fl}$*, *p48^{Cre}; p53$^{fl/fl}$; Tsc1$^{fl/+}$*mice. *p48^{Cre}; Tsc1$^{fl/+}$* mouse line shows no significant changes, while *p48^{Cre}; p53$^{fl/fl}$* mouse line shows an increased inflammation level followed by other associated activated pathway except for less apoptosis due the deficiency of p53. Interestingly in the *p48^{Cre}; p53$^{fl/fl}$; Tsc1$^{fl/+}$* mouse line, although there is no significant changes in ROS level, all other pathways are less activated compared to other groups.

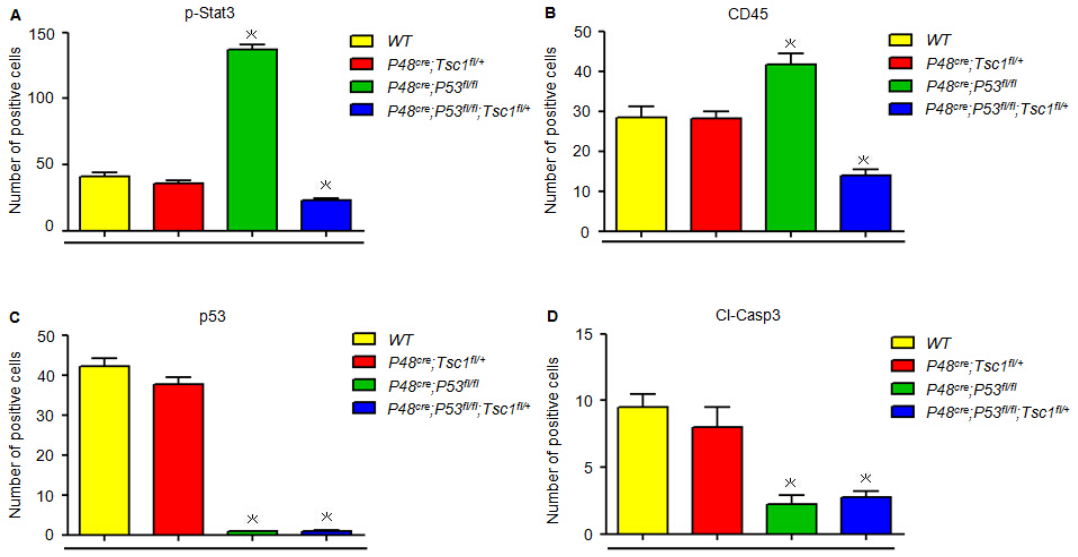

Figure19: A-D: Statistics data of IHC results of p-Stat3, p53, cleved-caspase-3 and CD45 for each group. (n=3, *$p<0.05$).

5. DISCUSSION

5.1. The establishment of an alcoholic chronic pancreatitis ROS generation mouse model

Reactive oxygen species, classified as a form of free radicals such as oxygen ions or non-radicals such as peroxides, is known to be mutagenic and plays a role in cancer formation (Shibutani et al., 1991). Briefly, the multi-stage cancer development process is characterized by the cumulative action of multiple events occurring in a single cell and can be described as three stages: initiation, promotion and progression during which each stage is influenced by ROS generation (Klaunig and Kamendulis, 2004). When ROS production and detoxification are not properly controlled in the cellular environment, the regulation of numerous oncogenes and tumor suppressor genes are impaired, which impinge on proliferation, apoptosis and cell migration, leading to cancer progression. Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive tumor, which has the highest case-fatality rate of any major cancer (Siegel et al., 2013). Although many studies have focused on PDAC, the etiology of PDAC is not yet fully understood. Numerous studies identified genetic changes and the role of these alterations in pancreatic cancer development. However, comparatively few research studies have focused on the role of inflammation and reactive oxygen species (ROS). Although many studies have examined the role of alcoholic chronic pancreatitis in the early initiation of PDAC, few have defined the role of ROS during this process. Thus, an ideal in vivo ROS generation mouse model is necessary to simulate in vivo conditions before tumor formation.

Indeed, multiple chronic pancreatitis mouse models have been available since the 1960s when Lieber and DeCarli performed a series of setups to investigate the

effects of ethanol on the liver (Lieber et al., 1965). Via the type of trigger used, we simply classified the mice as follows. The most popular models include ethanol feeding, repetitive caerulein injections and surgical ligation of the pancreatic duct as well as genetic models. Long term alcohol abuse commonly occurs and may lead to a persistent chronic inflammatory status to the pancreas (Apte et al., 2000); therefore, we typically used ethanol as our main experiment stimulus. To date, three primary ethanol-dependent mouse models have been introduced as follows:theintragastric ethanol infusion model (Tsukamoto et al., 2008); the "Lieber-DeCarli Formula" method (Kono et al., 2001) and the ethanol injection plus caerulein (Charrier and Brigstock, 2010). The intragastric ethanol infusion of mice is costly, labor-dependent and not readily amenable to most research studies. Furthermore, the ethanol-containing diet is time consuming and a less controlled approach; therefore, the daily precise i.p. injection into the mouse was introduced in our model. Based on the "the necrosis-fibrosis hypothesis" theory that the pathological changes of chronic pancreatitis result from recurrent acute pancreatitis that may not manifest itself clinically (Ammann et al., 1996; Apte et al., 2000; Apte and Wilson, 2003; Whitcomb, 1999), a single caerulein i.p. injection was also included in our model each week to generate recurrent acute pancreatitis.

The results of our mouse model perfectly met our aim according to our experimental results. Inflammation of the tissue was accomplished; both the gross and micro postacute pancreatitis morphology were confirmed by the loss of acinar cells, inflammatory infiltration, with some features of chronic pancreatitis, including areas of parenchymal fibrosis and build-up of stromal elements in our ethanol plus caerulein group. The liver from the treatment group appears be normal as shown by gross and micro morphological examinations. Notably, ROS, was also significantly generated in our mouse model.

Apparently, our model offers several advantages. First, our model reduces time

and costs. Our model can be examined within a three-week short time with a successful generation of ROS and sustained inflammation in pancreas. Second, our model is easily created. In the Lieber-DeCarli diet, feeding must be strictly monitored for caloric intake, specialized feeding equipment is required, and the intragastric ethanol infusion model needs a special operation on the mouse with a specialized infusion instrument, while our mouse model can simply accomplished by i.p. injection. Third, our model enables precise control. In the Lieber-DeCarli diet model, feeding must be strictly monitored, whereas the ethanol intake of our model can be exquisitely controlled by simple i.p. injection while the mice can still drink water and be fed ad libitum.

5.2. ROS generation on pancreatic biology

Recently, more studies have shown that the interaction between inflammation and ROS may play a contributory role in numerous pathologies, including early stage of pancreatic carcinogenesis (Goto et al., 1991; Greer and Whitcomb, 2009; Whitcomb, 2004). However, the precise mechanism of inflammation causing pancreatic malignancy is not well understood. Many theories illustrate the underling mechanism such as persistent chronic inflammation, NF-κB pathway activation, and the destabilized function of p53, which all involve ROS.

Here, we established an ACP ROS generation mouse model and examined the signaling pathway. We first discovered that sustained ethanol plus caerulein treatment causes chronic pancreatitis and generates high ROS levels. Furthermore, the Jak/Stat pathway was activated, which may worsen the inflammation level, and p53 and cleaved-capase-3 were also activated to regulate the ROS level. Recent studies on p53 signaling revealed that p53 affects ROS production partially by regulating the expression of antioxidant enzyme manganese superoxide dismutase (MnSOD) (Holley et al., 2010).The

redox-mediated regulation of p53 may be involved in controlling the cellular switch between survival and death in response to oxidative stress (Sun et al., 2013). Notably, our findings support this theory because p53 was activated in the ethanol plus cerulean group to cope with the high level of ROS generation. In addition, a higher SOD1, SOD3, GPX1 mRNA expression was also discovered to correspond to p53 activation. A sharply decreased expression of the MnSOD system enzymes in our results further confirm our hypothesis that p53 regulates ROS levels by activating the MnSOD anti-oxidant enzyme system.

Apoptosis was induced during inflammation and ROS generation. The apoptosis of the cell together with the inflammatory cytokine attracts the infiltration of other inflammatory cells, which may be another important endogenous source of ROS. However, apoptosis was completely blocked in the p53-deficient mouse line in our later experiment, indicating that ROS-mediated apoptosis may be p53-dependent. Inflammation, p53 and ROS work as a delicate loop that maintains a normal cellular fate.

Taken together, the results show that ROS levels may significantly contribute to inflammation. The inflammation level may also be mediated by cellular apoptosis. Thus, we propose that either blocking ROS generation or the inhibition of cell apoptosis may decrease the pancreas inflammation level.

5.3. Anti-inflammatory effects of antioxidant or anti-apoptosis treatment

Recently, studies have documented an important role of anti-oxidant treatment in mediating inflammatory responses in patients and mouse livers (Bae et al., 2013; Zhao et al., 2014). According to our previous results, we proposed that antioxidant treatment may mediate p53 activation and decrease the inflammation

level. Thus, butylated hydroxyanisole (BHA), widely known as an antioxidant preservative in food, food packaging, animal feed and cosmetics, was introduced in our mouse model. During the three-week treatment time, the mice ate specifically designed food containing BHA. The antioxidant function acted throughout the entire experimental procedure. Although the expression of the ROS markers, MDA and 8OHdG, only slightly decreased, the ROS serum and ROS scavenger levels showed that the BHA food decreased the ROS level. Markedly, both gross and micro morphology of BHA-treated mice appeared nearly normal. The IHC staining of p-stat3, cleaved-caspase-3 and CD45 all significantly decreased. p53-positive staining slightly decreased. The results further confirm our hypothesis that ROS is important in mediating inflammation.

Here, based on the results, ethanol plus caerulein activates the Jak/Stat pathway, and ROS further induces the Jak/Stat pathway. Upon the stimulation of ROS, p53 is activated to mediate the stress tension, and the anti-ROS system is simultaneously activated, including the p53/caspase apoptosis pathway and the MnSOD ROS scavenger system. Thus, via p53 activation, the cellular ROS level is maintained as a normal value.

Regarding the association between inflammation and apoptosis, the current data in the literature are controversial. Severe inflammation induces cellular apoptosis via several cell death pathways such as the p53-dependent caspase pathway or the Bax/Bcl-2 pathway. However, whether further interactions occur between apoptosis and inflammation remain unknown. Interestingly, in our study, when the downstream target of the p53/caspase pathway was inhibited by the caspase-3 specific inhibitor, Z-DMVD-FMK, the cell death findings and HE staining presented better gross and micro morphology. The CD45 staining was significantly less, and the level of the serum inflammation marker decreased. p53 activation and p-stat3 were not affected, and the ROS level remained the same as after treatment, which may be due to the inhibition of apoptosis, which decreases

inflammatory cell infiltration. Less cytokines are released into the stoma, inducing less inflammation. However, further investigations need to be performed.

5.4. Inactivation of the Tsc/mTORC1/p53 signal loop promotes ROS generation and inflammation

Based on the p53 activation observed in our ACP mouse, the role of p53 in mediating ROS generation and inflammation will be further studied. Nevertheless, how ROS modulates the selective transactivation of p53 target genes is a challenging question. As a tumor suppresser, one of the major functions of p53 is to restrict abnormal or stress-exposed cells before excessive DNA damage is caused by ROS (Lane, 1992). Thus, a p53-deficient mouse line was generated in our research. mTOR has emerged as a key regulator for cellular metabolism and plays an important part in the physiology of various organs (Chen et al., 2008). Recently, up-regulated mTOR activity is associated with a higher level of intracellular reactive oxygen species (ROS) (Jang and Sharkis, 2007). Tsc is the important major negative regulator of mTOR (Giaccone et al., 1990; Melnik and Schmitz, 2013); therefore, Tsc1 haploinsufficient mice were also included in our study. The Tsc/mTORC1/p53 signal loop is a delicate feedback system; therefore, p53 was also inactivated in the context of Tsc1 haploinsufficiency to generate a *p48^{Cre}; p53$^{fl/fl}$; Tsc1$^{fl/+}$* mouse line to determine the interactions between each axis of the Tsc/mTORC1/p53 loop.

To date, quite beyond our expectation, after a three-week treatment, although the histological examination of pancreases obtained from the Tsc1-deficient mice revealed an inflammation phenotype in the ethanol plus caerulein treatment group compared with the water and saline group, no obvious difference was observed in the morphology in Tsc1-deficient mice compared with the double treatment *WT* mice. ROS levels were slightly lower than in the *WT* group, and the p-Stat3, p53, cleaved-caspase-3 levels were also slightly decreased. In addition, the partial

deletion of Tsc1 did not appear to harm mTOR signaling. IHC results revealed that only slightly mTOR increased expression occurred compared with*WT* mice. To conclude, Tsc1 haploinsufficiency had no significant impact on pancreatic physiology. In contrast, when p53 activity is completely blocked, the mouse pancreas presents significant morphological and biological changes after sustained treatment. Notably, p53 inactivation appears to strongly disturb the Tsc/mTORC1/p53 signal loop, and high ROS and p-mTOR$_{s2448}$ were observed in the p53-deficient mice compared with the double treatment *WT* mice. An increased ROS generation was associated with increased inflammatory cells and strong Jak/Stat pathway activation compared with the other groups. p53 deficiency may lead to a disturbed antioxidant system as well as an uncontrolled mTOR level, which causes high persistent ROS levels. Under these circumstances, the Jak/Stat pathway is highly activated, which also contributes to severe inflammation. These findings highlight the role of p53 activation in mediating metabolism and oxidative/redox stress. However, when the function of p53 and Tsc1 was impaired, the results were surprising. The activity of all he signaling pathways, including Jak/Stat, p53, mTOR, and inflammation, were significantly lower. The gross and micro morphology appeared normal tissue. The tissue appeared to be not be affected by the environmental stresses; therefore, underlying mechanism needs to be further examined.

6. SUMMARY

This study developed a quick, efficient and cost-effective alcoholic chronic pancreatitis ROS generation model to examine the interaction between ROS generation/degradation and tissue inflammatory response. In addition, transgenic mouse lines were utilized to identify potential genes involved in the mediation of these delicate processes.

In summary, our ACP mouse model successfully generated ROS and inflammation. Our study demonstrates that a highly relevant partnership between high ROS levels may indicate high inflammation levels because antioxidant treatment successfully promotes the inflammation status. Apoptosis may also be involved in the inflammatory response because the caspase-3 inhibitor reduces the inflammation level. In addition, p53 plays an important role in maintaining the ROS level in which the antioxidant enzyme activated by p53 as well as mTOR signaling may be partly involved.

In addition, the questions raised in the “AIMS OF THIS STUDY” section have been addressed as follows:

Question 1 (Q1): Is ROS upstream of inflammation or a downstream target of inflammation? What are the effects on inflammation if ROS is artificially removed? Conclusion 1 (C1): ROS may be the most important byproduct of inflammation, which may then acts as a mediator of inflammation. A high level of ROS may lead to a worse inflammatory response. ROS may worsen inflammation via activation of the Jak/Stat pathway, which releases inflammatory cytokines such as IL-6, IL-1β, and MCP-1. In contrast, cell apoptosis caused by p53activation as a result of high ROS level may worsen the inflammation.

Question 2 (Q2): How does p53 regulate and control ROS levels?

Conclusion 2(C2): Firstly, p53 is activated by high ROS levels, and the MnSOD p53-associated cellular anti ROS enzyme system is then activated to mediate the ROS level. In addition, p53 activation also maintains the ROS balance via Tsc/mTORC1/p53 feedback signal loop.

Question 3 (Q3): Does apoptosis affect tissue inflammation levels?

Conclusion 3(C3): Our study confirmed that inflammation may be caused or be worsened by apoptosis. ROS may also be involved in the process.

Question 4 (Q4): What will occur to ROS or inflammation if either nude or Tsc/mTORC1/p53 is impaired?

Conclusion 4(C4): The Tsc/mTORC1/p53 axis is an important feedback signaling loop. However, Tsc1 haploinsufficiency shows no impact on pancreatic physiology. While p53 deficient may lead to both high ROS and inflammation levels, the combination of Tsc1 haploinsufficiency and p53 loss do not affect the normal physiology of the pancreas.

7. ABBREVIATIONS

TP53BP1	Tumor protein p53 bindingprotein 1
8OHdG	7,8-dihydro-8-oxo-2'deoxyguanosine
Ab	Antibody
ADM	Acinar-to-ductal metaplasia
AMPK	AMP-activated protein kinase
ATM	Ataxia telangiectasia mutated
BSA	Bovine serum albumin
Casp3	Caspase 3
CAT	Catalase
CCK	Cholecystokinin
CD45	leukocyte common antigen
Cre	Crerecombinase
CSC	Cancer stem cell
DAPI	4,6-diamidino-2-phenylindole
D	Day
DNA	Deoxyribonucleic acid
ERK	Extracellular signal-regulated kinase
ER	Endoplasmic reticulum
FAEE	Fatty acid ethyl ester
FAEEs	Fatty acid ethyl ester synthase
GAP	GTPase Activating Protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GEF	Guanine-nucleotide exchange factor
GEMM	Genetically engineered mouse model
GPX	Glutathione peroxidase
GRB10	Growth factor receptor-bound protein 10
GSK3	Glycogen synthase kinase 3
GSH	Glutathione
GSSH	Glutathione
GWAS	Genome-wide association study
HCC	Hepatocellular carcinoma
HIF1A	Hypoxia inducible factor 1, α subunit (basic helix-loop-helix transcription factor)
HRP	Horseradish peroxidase
HSCs	Hematopoietic stem cells
IHC	Immunohistochemistry
IKK2	Inhibitor of kappaB kinase β
LI1β	Interleukin 1 β
IPMN	Intraductal papillary-mucinous neoplasm
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral

	oncogene homolog
Krt19	Keratin 19
LKB1	Serine/threonine kinase 11
MDA	Malondialdehyde
MEK	Mitogen-activated protein kinase kinase
MnSOD	Manganese superoxide dismutase
mTOR	Mammalian target of rapamycin
NADH	Reduced form of nicotinamide-adenine dinucleotid
NADPH	Dihydronicotinamide adenine dinuclectide phosphate
NF-κB	Nuclear factor of kappa light polypeptide gene enhancer in B cells 1
p48	Pancreas specific transcription factor, 1a
p53	Tumor protein p53
PanIN	Pancreatic intraepithelial neoplasia
PARP1	Poly (ADP-ribose) polymerase 1
PBS	Phosphate buffered saline
PCA	Principal component analysis
PDAC	Pancreatic ductal adenocarcinoma
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
ROS	Reactive oxygen species
S6	Ribosomal protein S6
S6K1	S6 kinase 1
SD	Standard deviation
SDS	Laurylsulfate
SH3 domain	SRC Homology 3 Domain
SMAD4	SMAD family member 4
SOD	superoxide dismutase
Stat3	signal transducer and activator of transcription 3
STE	Sodium chloride and TE
TBARS	Thiobarbituric Acid Reactive Substances
TP53INP1	Tumor protein p53-induced nuclear protein 1
TSC	Tuberous sclerosis
YY1	Yin-yang 1 protien
α-SMA	A-smooth muscle actin

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